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Reviews

PERI-OPERATIVE ANALGESIC EFFICACY OF TRAMADOL IN DOGS AND CATS

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SUMMARY

Tramadol, classified as an 'atypical opioid', is a centrally acting analgesic that is easily available in injectable and oral preparations in Malaysia. Queries on its use and efficacy to manage pain in small animal practice prompted this review. Following tramadol administration in dogs, there is lack of sedation and opioid effects when compared to cats. This may likely due to species difference in the production, metabolism and elimination of O-desmethyltramadol (M1). Good efficacy of tramadol was reported in both dogs and cats undergoing ovariohysterectomy and castration. Pre-emptive doses were administered via parenteral routes between 2 – 4 mg/kg, usually during premedication or after induction. Adequate pain control was reported with tramadol 2 mg/kg, SC every 8 hours following maxillectomy or mandibulectomy in dogs. Thus, adequate analgesia can be promoted with increase doses or repeated parenteral dosing following orthopaedic procedures. Repeated oral doses of tramadol as the sole analgesic were found to be less effective in dogs undergoing tibia plateau levelling osteotomy. Combinations of tramadol and a non-steroidal anti-inflammatory drug were found to provide better post-operative analgesia in both dogs and cats.

Keywords: analgesic, cats, dogs, pain, tramadol

INTRODUCTION

Tramadol is widely available in both injectable and oral preparations in Malaysia, and sold under trade names such as Acugesic, Analab, Mabron, Pengesic, Tracidol, Tramox, and Tramadol Stada, to name a few. It is listed under the first schedule of the Poisons List (Section 2) under Poison Act 1952 (Revised 1989) in Malaysia. With the limited choice of analgesics available to veterinarians in Malaysia, tramadol may be a popular option and plays important role in pain management. In MIMS, the indication for use of tramadol is moderate to severe pain in humans. In small animal practice, there had been queries and different opinions on the clinical efficacy of tramadol. This article aims to review selected literatures and clinical studies, with focus on the peri-operative analgesic efficacy of tramadol in dogs and cats.

Mechanism of action

Tramadol is a centrally acting analgesic, structurally related to codeine and morphine (Lewis and Han, 1997). It is an μ-opioid receptor agonist, however, its affinity for the μ, δ, and κ is weak. Tramadol also inhibits the reuptake of noradrenaline and serotonin in the central nervous system, thereby enhancing the descending pain inhibitory system to modulate pain. This explains why the analgesic effect of tramadol was only partially antagonised by naloxone (Raffa *et al*., 1992). Due to its dual action on the opioid and the non-opioid systems, tramadol is classified as an "atypical opioid".

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Pharmacokinetic studies of tramadol in dogs

Tramadol can be metabolised into at least 30 different metabolites, but only tramadol, O-desmethyltramadol (M1), and N,O-didesmethyltramadol (M5) have been associated with pharmacologic effects (Grond and Sablotzki, 2004). The opioid effects following tramadol administration is primarily contributed by M1, which is 200 times more potent than tramadol in binding of the μ-receptor (Raffa *et al.*, 1992). Due to its high affinity to the μ receptors, an intravenous (IV) injection of only 1 mg/kg M1, can caused nausea and sedation in beagle dogs (KuKanich and Papich, 2004). However, M1 production is slow in dogs, and its elimination is faster than its production (Mc Millan *et al.*, 2008). The relatively low plasma concentration of M1 in dogs may explain the lack of opioid effect following tramadol administration in this species. Furthermore, tramadol is more rapidly degraded to inactive metabolites in dogs (KuKanich and Papich 2004, Mc Millan *et al.*, 2008) compared to humans (Grond and Sablotzki, 2004), thereby affecting its efficacy in dogs.

Pharmacokinetic study using 4 mg/kg tramadol via the IV and intramuscular (IM) route showed that absorption was rapid after IM injection (Giorgi *et al.*, 2010). Bioavailability was 92 +/- 9% and peak plasma concentration was reached in 0.34 hours after IM injection (Table 1). The high bioavailability makes the IM route a useful alternative to the IV route in dog. Data in this study showed that both the IV and IM administration of 4 mg/kg tramadol can achieve the minimal effective concentrations associated with analgesia in humans within 1.1 +/- 0.2 minutes and lasted for 6-7 hours.

Bioavailability of tramadol following the oral route was reported as 65 +/- 38% by KuKanich and Papich (2004). Large inter-individual variability was observed with this route. Simulated oral dosing predicted that 5 mg/kg every 6 hours, and 2.5 mg/kg every 4 hours would

Table 1. Pharmacokinetics of tramadol by routes of administration

Route (dose rate)	Bioavailablity (%)	Volume of distribution at steady state (mL/kg)	Time to reach peak plasma concentration (hrs) T_{max}	Peak plasma concentration (ng/ml) C_{max}	Half life of the elimination phase (hrs) $t_{1/2\alpha}$
[1]Intravenous (4mg/kg)	--	1003±472	--	--	0.42±0.39
[1]Intramuscular (4mg/kg)	92±9	--	0.34±0.05	252±0.43	0.73±0.16
[2]Oral (9.9 ± 0.4 mg/kg)	--	--	*1.3	*215.7	--
[3]Rectal (4mg/kg)	10.03±4.52	--	0.56± 0.41	140±60	2.24±1.82
[4]Extradural (2mg/kg)	82	--	1.15±0.31	0.18±0.12	--
[5]Intranasal (4mg/kg)	20.6	--	0.67±0.22	123.17±46.33	--

[1]Giorgi *et al*., 2010; [2]KuKanich & Papich, 2011; [3]Giorgi *et al*., 2009; [4]Vettorato *et al*., 2010; [5]Di Salvo *et al*., 2020; *geometric mean

Table 2. Studies on the efficacy of tramadol in dogs

Study design	Anaesthetic and analgesics	Parameters assessed	Results
[1]Clinical 30 dogs with pyometra n = 15/group MORP (morphine) TRA (tramadol)	ACE, ketamine, midazolam isoflurane MORP 0.2 mg/kg, IV or TRA 2 mg/kg, IV Rescue analgesia: MORP	SDS VAS catecholamine cortisol glucose	No group difference in SDS, VAS, sedation, SpO2, pH, blood gases, cardiovascular variables, catecholamine, cortisol and glucose More respiratory depression in MORP EtIso at 40 min: MORP (0.8 ± 0.3%); TRA (1.0 ± 0.3%) Number of dogs required rescue analgesia: MORP (1), TRA (2)
[2]Clinical: 42 dogs maxillectomy or mandibulectomy 5 groups TRA (tramadol, n=8) COD (codeine, n=9) KETO (ketoprofen, n=9) TRA + KETO (n=8) COD + KETO (n=8)	ACE, pethidine propofol, isoflurane Test analgesic start 30 mins before surgery ended, SC route: TRA 2 mg/kg, q8h COD 2 mg/kg, q8h KETO 2 mg/kg, q24h TRA 2 mg/kg, q8h + KETO 2 mg/kg, q24h COD 2 mg/kg, q8h + KETO 2 mg/kg, q24h <u>Rescue analgesia</u> Metamizole, MORP	NRS SDS sedation IL-6, cortisol glucose	No group difference in NRS No significant change in IL-6 and cortisol Glucose increased in all groups except TRA + KETO Number. of dogs required rescue analgesia: KETO (5); TRA (2); COD (2) TRA + KETO (2); COD + KETO (2) All groups provided adequate analgesia Light sedation in COD + KETO at 4, 5, 24 h

[3]Castration n= 8/group MORP (morphine) TRA (tramadol)	ACE, atropine thiopental, halothane Premedication: MORP 0.5 mg/kg, SC or TRA 3 mg/kg, SC	EEG parameters (F50), total power (Ptot), 95% spectral edge frequency (F95%) CMPS-SF	Higher F50 and lower Ptot in TRA during ligation of 1st testicle. No treatment difference on 2nd testicle. No treatment difference in F95. No treatment difference in CMPS-SF during the 9 h post-op study. No dog required rescue analgesia. Dogs in MORP more sedated pre-operatively and 1 h post-op.
[4]Clinical: OHE 75 dogs n=25/group DKETO (dexketoprofen) BUP (buprenorphine) TRA (tramadol)	Medetomidine, 3 ug/kg, SC. propofol, isoflurane DKETO 1 mg/kg, SC, TID or BUP 0.02 mg/kg, IV, TID or TRA 2 mg/kg, IV, BID Rescue analgesia: MORP 0.2 mg/kg IM	DIVAS CMPS-SF	DIVAS: BUP > DKETO and TRA at 2 and 4 h CMPS-SF: BUP > DKETO and TRA at 2, 4, 6 and 18 h Number of dogs require rescue analgesia: BUP (10/23, 43%), TRA (5/23, 21%), DKETO (1/20, 5%)
[5]Clinical: TPLO 30 dogs n= 10/group TRA (tramadol) FIRO (firocoxib) TRA+FIRO	ACE, MORP 1 mg/kg, IM, propofol, isoflurane HYDRO 0.05 mg/kg, SC, q6h *TRA 4-5 mg/kg, PO, TID or *FIRO 5 mg/kg, PO, SID or *TRA + FIRO *start evening prior to surgery Rescue analgesia: HYDRO 0.05 mg/kg, SC	GCMPS cortisol limb function	Number of dogs with GCMPS > 6: TRA (8) > TRA+FIRO (6) > FIRO (4) Number of dogs with GCMPS > 8; required rescue analgesia: TRA (4) > FIRO (1) = TRA+FIRO (1) Limb function: TRA: Day 1, 2 < baseline FIRO: Day 1 < baseline TRA+ FIRO: reduction not significant No group difference in cortisol changes
[6]Clinical: TPLO 3 groups n= 9 dogs/group TRA (tramadol MTD0.5 (methadone 0.5 mg/kg) MTD0.7 (methadone 0.7 mg/kg)	Propofol, isoflurane fentanyl 3 µg/kg, once, before osteotomy Pre-medication: TRA (4 mg/kg, IM) or MTD0.5 (0.5 mg/kg, IM) or MTD0.7 (0.7 mg/kg, IM) Rescue analgesia: TRA 1 mg/kg or MTD: 0.2 mg/kg	VAS GCMPS CUCAPS sedation IL-6	VAS: TRA and MTD0.5 > MTD0.7 at post-4 h Within TRA, post-4 h > baseline, post-1, post-24 h post-1 h >post-6 h Within MTD0.5, post-4 h > post-1 h GCMPS: MTD0.7 < TRA and MTD0.5 at post-4 h Number of dogs required rescue analgesia: TRA (6/9)> MTD0.5 (1/9), MTD0.7 (0/9) No time nor group difference in sedation, CUCAPS, IL-6 *TRA- nausea, intense salivation (n=8)

[7]Clinical: TPLO 2 groups	MORP (0.3-0.5 mg/kg, SC) or HYCD (0.01-0.06 mg/kg, SC) midazolam, propofol, isoflurane, intraarticular bupivacaine,	GCMPS	GCMPS higher in TRA at 2 h after 2nd oral dose; no difference at other times. No difference in number of dogs requiring rescue analgesia: TRA (7/23); HYCD (5/19).
TRA (tramadol, n=23)	MORP (0.25-0.5 mg/kg, SC)		*TRA – regurgitation in 6 dogs, 1 required treatment
HYCD (hydrocodone-acetaminophen, n= 19)	Post-op test analgesics: TRA (5-7 mg/kg, PO, TID) or HYCD (0.5-0.6 mg/kg, PO, TID) Rescue analgesia: MORP (0.25 -0.5 mg/kg, SC)		

ACE= acepromazine; COD = codeine; CO_2= carbon dioxide; CMPS-SF = short form of Glasgow composite measure pain scale; CPS = composite pain score; CUCAPS = Colorado University canine acute pain scale; DIVAS = dynamic interactive visual analogue scale; DKETO = dexketoprofen; FIRO = firocoxib; GCMPS = Glasgow composite measure pain scale; h = hour; HYCD = hydrocodone; HYDRO = hydromorphone; IM = intramuscular; IV = intravenously; KETO = ketoprofen; min= minutes; MORP = morphine; OHE = ovariohysterectomy; SC = subcutaneously; SDS = simple descriptive scale; TPLO = tibial plateau leveling osteotomy; TRA = tramadol; VAS = visual analog scale

[1]Sandra *et al*., 2003; [2]Martins *et al*., 2010; [3]Kongara *et al*., 2013; [4]Morgaz *et al*., 2013; [5]Davila, *et al*., 2013; [6]Cardozo *et al*., 2014; [7]Benitez *et al*., 2015

achieve plasma concentrations of tramadol and M1 associated with analgesia in humans. The same authors reported significant increases in pressure thresholds only at 5 and 6 hours after an oral dose of 10 mg/kg tramadol in Greyhounds (KuKanich and Papich, 2011). The plasma concentration of M1 was < 1 ng/ml at 6 hours, when the antinociceptive effects peaked. Such low concentration of M1 have not been shown to be analgesic. Thus, the authors suggested that the antinociceptive effects in Greyhounds may be independent of M1 plasma concentration.

Study on tramadol administered via the extradural (ED) route at 2 mg/kg in dogs undergoing tibial plateau levelling osteotomy (TPLO) revealed pharmacokinetic profile that was similar to the IV route (Vettorato *et al*., 2010). In this study, the ED route provided sufficient intra- and post-operative analgesia, but the effect was not superior to the IV route. Thus, the authors concluded that the ED route is not a practical alternative to the IV route. Administration of tramadol at 4 mg/kg via the rectal route revealed bioavailability of only 10 +/- 4% (Giorgi *et al.*, 2011). Absorption was rapid, and so was the metabolism of tramadol to M2 and M5. The poor availability and extensive biotransformation of tramadol to inactive metabolites suggest limited utility of this route.

Recently, the intranasal (IN) route using 4 mg/kg tramadol was attempted in bitches undergoing ovariohysterectomy (Di Salvo *et al*., 2020). The peak plasma concentration was low (74 - 200 ng/ml), and can be detected in the plasma for only 2 hours post-IN administration. However, the analgesic effect during the 8 hours of post-OHE study was comparable to dogs given tramadol 4 mg/kg, IV, or methadone 0.2 mg/kg, IV. These results corroborate with the hypothesis of a direct nose-to-brain pathway for tramadol, and may be a potentially useful route.

Studies on the efficacy of tramadol in dogs

The post-operative analgesia effect of tramadol has been demonstrated in dog undergoing ovariohysterectomy (Morgaz *et al.*, 2013; Sandra *et al.*, 2003) and castration (Kongara *et al.*, 2014). Tramadol at 2 mg/kg, IV was reported to be as effective as morphine (0.2 mg/kg, IV) to control early pain following OHE in dogs with pyometra (Table 2). In this study, there were no treatment difference in pain score, sedation, glucose, catecholamine, cortisol, SpO_2, blood pH and cardiovascular variables (Sandra *et al.*, 2003). End-tidal CO_2 at 30 mins post-tramadol injection was significantly lower, suggesting less respiratory depression than morphine.

Premedication with tramadol at 3 mg/kg, subcutaneously (SC), was shown to provide similar post-op analgesia as compared to morphine (0.5 mg/kg, SC) for castration in dogs (Kongara *et al.*, 2013). However, tramadol is less effective in preventing changes in the intra-operative EEG indices of nociception during surgery, especially during ligation of the first testicles.

A clinical study that utilised repeat dosing in a 48-hour post-OHE study also showed that tramadol [2 mg/kg, IV, two times a day (BID)] provided superior analgesia compared to buprenorphine [0.02 mg/kg, IV, three times a day (TID)] in dogs (Morgaz *et al.*, 2013). In

this study, pain scores in the tramadol group is lower than the buprenorphine group, but comparable to the dexketoprofen group. The failure rate of this tramadol regimen was 21%, compared to 43% and 5% in buprenorphine and dexketoprofen respectively; and these occurred mainly in the first 2 - 4 hours post-OHE.

Review on the use of tramadol on orthopaedic procedures returned mixed results, depending on procedures and dose regimes. Tramadol (2 mg/kg, SC) administered 30 minutes before end of maxillectomy or mandibulectomy, and repeated every 8 hours provided analgesia comparable to codeine, 2 mg/kg, SC, every 8 hours (Martin *et al*., 2010). In dogs undergoing tibial plateau leveling osteotomy (TPLO), premedication with tramadol (4 mg/kg, IM) resulted in higher pain scores and rescue analgesia requirement compared to methadone at 0.7 mg/kg, IM (Cardozo *et al*., 2014). Rescue analgesia at only 1 mg/kg tramadol could abate signs of pain, and morphine was not required. Therefore, increase doses or repeated doses of injectable tramadol could improve pain control.

Repeated oral doses of tramadol alone (4 – 5 mg/kg, TID) were found to be inferior compared to firocoxib, or their combinations in providing analgesia to dogs post-TPLO (Davila *et al*., 2013). In another study, pain scores and rescue analgesia requirements in dogs given tramadol (5 – 7 mg/kg, PO, TID) were not different from hydrocordone-acetaminophen (Benitez *et al*., 2015a). The treatment failure rate of 30.4% in tramadol, and 29% in hydrocordone was considered unacceptable. Wide variations in the serum concentration of tramadol, and low M1 concentration (Benitez *et al*., 2015b) may have contributed to the observed efficacy. Thus, it is may not be advisable to use oral tramadol as the sole analgesic following orthopaedic procedures. Based on the principle of multimodal analgesia, incorporation of a non-steroidal anti-inflammatory drug, if not contra-indicated, may manage pain better.

Studies of tramadol in cats

In contrast to dogs, there are less published studies of tramadol in cats. Studies evaluating the antinociceptive effects of tramadol in cats are summarised in Table 3. In 2007, Chen and colleagues have reported that the addition of tramadol at 4 mg/kg, SC during premedication provided better analgesia for up to 8 hours post-ovariohysterectomy (OHE). In this study, control group that received only tolfenamic acid, 4 mg/kg, SC at the end of surgery recorded higher pain scores at 3, 4, 6 and 8 hours post-OHE. Further, there were no differences in the pre- and post-operative creatinine and alanine aminotransferase in either groups.

Comparison between repeated dose of tramadol 3 mg/kg, SC versus tolfenamic acid, 4 mg/kg, SC revealed lower pain scores in the tramadol group at 4 and 8 hours post-OHE (Tan *et al*., 2009). In this study, tramadol was administered at premedication and repeated 10 hours later, while tolfenamic acid at end of surgery and repeated 24 hours later. These results showed that pre-emptive tramadol confer better analgesia in the early post-operative periods. However, all post-OHE mechanical thresholds determined near the surgical site (MTs) were lower than baseline values. This indicates that the development of primary hyperalgesia was not completely reversed by either use of repeated tramadol or tolfenamic acid alone.

Further dose reduction of tramadol to 2 mg/kg, SC resulted in higher post-OHE pain score and more decrement of MTs (i.e. hyperalgesia) compared to tramadol 4 mg/kg, SC (Basiri *et al*., 2014). In this study, all cats in the negative control group that received only acepromazine in the premedication required rescue analgesia, while none in the tramadol groups needed rescue analgesia. Results from this study further confirm that acepromazine lack analgesic effect and tramadol provided dose-dependent analgesia in early post-operative periods.

Experimental study with tramadol at 1 mg/kg, SC showed limited effect on the thermal and mechanical nociceptive thresholds but can be enhanced by the addition of acepromazine at 0.1 mg/kg, SC (Steagall *et al*., 2008). However, tramadol at 1 mg/kg, via the epidural route was shown to provide analgesia comparable to epidural morphine at 0.1 mg/kg for up to 6 hours, based on a tail-clamp noxious stimulation (Castrol *et al*., 2009). In applying into practice, readers are advised to use preservative-free preparations of tramadol for the epidural injection to avoid risk of preservative or excipient related neurotoxicity.

The pharmacokinetics and disposition of tramadol and its major metabolite, M1, have been studied after intravenous (IV) and oral (PO) administration in awake cats (Pypendop and Ilkiw, 2008). The volume of distribution of tramadol following IV or PO in cats is similar to dogs, while its clearance is lower, resulting in longer elimination half-life in the cats (Pypendop and Ilkiw, 2008; KuKanich and Papich, 2004). Higher inter-individual variability in the tramadol disposition was observed in the oral route, most likely explained by differences in the individual cat's absorption rate and gastro-intestinal transit time.

Following both IV and PO tramadol in cats, M1 appeared rapidly in the plasma and remained high. The area under the curve (AUC) for M1:tramadol was found to be close to 1 in cats, compared to 0.3 in dogs (KuKanich and Papich, 2004). The higher AUC of M1 in cats may indicate higher production of M1 in cats, or it may be due to slower glucuronidation, leading to slower elimination. The higher AUC of M1 in cats most likely explains the more prominent opioid effect seen in cats compare to dogs.

In another related study by Pypendop *et al*. (2009), the plasma concentrations of tramadol and M1 were found to be dose proportional from 0.5 to 4 mg/kg via the oral route. In this study, a dose-dependent thermal antinociceptive effect that lasted up to 6 hours was demonstrated at doses of 2, 3 and 4 mg/kg, PO. Pharmacokinetic simulation from this study predicted that a dose of 4 mg/kg, every 6 hours, PO, would maintain adequate analgesia in cats.

The pharmacokinetic parameters from cats treated with tramadol, 2 mg/kg, IV, and subjected to gonadectomy under isoflurane anaesthesia (Cagnardi *et*

Table 3: Studies evaluating tramadol in cats

Study design	Anaesthetics and analgesics)	Parameters assessed	Results
[1]Clinical: Midline OHE 2 groups n = 6/group	ACE 0.1 mg/kg, SC, thiopental, halothane TOLF 4 mg/kg, SC, end of surgery Premedication: CTRL: ACE TRA: ACE + TRA 4 mg/kg, SC	CPS Serum creatinine, ALT	CPS: CTRL > TRA at 3, 4, 6, and 8 h Within CTRL: 3, 4, 6, 8, 24 h > baseline. Within TRA: 24 h > baseline. No change in pre- and post-op creatinine and ALT in both groups.
[2]Experimental: 8 cats	Sal 0.3 ml, SC TRA 1 mg/kg, SC ACE 0.1 mg/kg, SC TRA + ACE, SC	TT MT	TRA had limited effect on TT and MT
[3]Experimental: 6 cats	Epidural administration of: CT: 0.22 ml/kg saline TT: 1 mg/kg tramadol MT: 0.1 mg/kg morphine	SDS VAS skin clamp	SDS, VAS score: CT > TT and MT at all times TT > MT at 8, 10, 12 h Euphoria, persisted up to 12 h: MT (5 cats), TT (4 cats)
[4]Experimental: 6 cats	TRA at 0.5, 1, 2, 3, 4 mg/kg, PO	TT	Thermal threshold higher than baseline at 80 and 120 mins for 0.5 mg/kg dose; 80 and 120-360 mins for 2 mg/kg dose; 40-360 mins for 3 mg/kg dose; and 60-360 mins for 4 mg/kg dose.
[5]Clinical: Midline OHE 2 groups n = 7/group	ACE 0.1 mg/kg, SC, thiopental, halothane TOLF 4 mg/kg, SC at extubation, repeat at 24 h TRA 3 mg/kg, SC, at premedication; repeat at 10 h	VAS MTp	VAS: TRA < TOLF at 4, 8 h MTp: No treatment difference All post-op values < baselines
[6]Clinical: OHE 4 groups n = 10/group	VEDA 0.5 mg/kg, PO q 24h TRA 2 mg/kg, SC q 8 h VEDA + TRA Placebo Treatment at 1 h pre-op and repeated until 72 h	IVAS CPS VFF	Number of cats required rescue analgesia: Placebo (10); VEDA (10); TRA (5); VEDA + TRA (0) Only VEDA + TRA prevented hyperalgesia No changes in pre- or post-op platelet aggregation, bleeding time, urea, creatinine, ALT, AlkPhos, GGT
[7]Clinical: 6 female, ovariectomy 6 male, orchiectomy	Atropine + ACE 0.05 mg/kg, IM Isoflurane (induce & maintain) TRA 2 mg/kg, IV over 15 s	Subjective pain sore PK study	Reduced requirement of isoflurane: Female (1.5+/-0.4%), Male (1.3+/-0.3%) Low post-op pain score, rescue analgesia not required (6 h study) No sex difference in pain and PK parameters

[8]Clinical: Midline OHE 3 groups n = 6/group	ACE 0.1 mg/kg, SC thiopental, isoflurane A: Sal AT2: TRA 2 mg/kg, SC AT4: TRA 4 mg/kg, SC	CPS MTs MTp	CPS: A> AT2> AT4 Number of cats required rescue analgesia: A (3/3)*; AT2 (0/6); AT4 (0/6) *stop recruitment for A at n=3 MTp : AT4> AT2> A Post-op MTs < baselines in all cats Least decrements in AT4
[9]Clinical: OHE 3 groups n = 14/group	ACE 0.1 mg/kg, IM, Propofol, Isoflurane PET 6 mg/kg, IM TRA 2 mg/kg, IM TRA 4 mg/kg, IM	CPS Serum glucose, cortisol, IL-6	CPS – no group difference within 6 h study Number of cats required rescue analgesia: PET (5); TRA2 (2); TRA4 (0) Cortisol: PET > TRA2 and TRA4 at 6 h Within PET: 1, 3, 6 h > baseline

ACE = acepromazine; CTRL = control; CPS = composite pain score; VFF = von Frey filament; h = hour; IVAS = interactive visual analog scale; MT = mechanical threshold; MTp = mechanical threshold at metatarsal pad; MTs = mechanical threshold near surgical site; OHE = ovariohysterectomy; PK = pharmacokinetic; Sal = saline; SC = subcutaneously, TOLF = tolfenamic acid; TRA = tramadol; TT = thermal threshold; VEDA = vedaprofen

[1]Chen *et al*., 2007; [2]Steagall *et al*., 2008; [3]Castro *et al*., 2009; [4]Pypendop *et al*., 2009; [5]Tan *et al*., 2009; [6]Brondani *et al*., 2009a,b; [7]Cagnardi *et al*., 2011; [8]Basiri *et al*., 2014; [9]Evangelista *et al*., 2014

al., 2011), was comparable to the study on awake cats (Pypendop and Ilkiw, 2008). The lower clearance of tramadol in this study may be related to the anaesthesia and surgery. Intra-operatively, cardiorespiratory parameters were within normal limits, with no respiratory depression noticed. Previously, tramadol at 2 and 4 mg/kg, IV have been reported to depress ventilation in chloralose-urethane anaesthetised cats (Teppema *et al.*, 2003). Lack of respiratory depression in this study may be due to the low level of isoflurane (1.4 +/- 0.4%) used in this study, which is below the minimum alveolar concentration for cats.

Comparative study on repeated doses of tramadol 2 mg/kg, SC, every 8 hours, or vedaprofen 0.5 mg/kg, PO, every 24 hours, or their combination, or placebo, revealed that the combination provided the most effective analgesia post-OHE (Brondani *et al*., 2009a). Cats that received both tramadol and vedaprofen had lower pain scores compared to cats administered either drugs on its own. Rescue analgesia were required by all cats in the placebo and vedaprofen groups, 50% in the tramadol group and none in the combination group. Pre- and post-OHE platelet aggregations, bleeding time, urea, creatinine, ALT, ALP and GGT in all groups did not alter (Brondani *et al*., 2009b), suggesting that peri-operative repeated use of these drugs should be safe.

Another clinical study using OHE as the pain model compared analgesia of pre-operative pethidine 6 mg/kg, tramadol 2 mg/kg, and tramadol 4 mg/kg, IM (Evangelista *et al*., 2014). Post-operative pain scores within the 6 hours post extubation were not different amongst groups. However, rescue analgesia was required by 5 out of 14 cats in the pethidine group, compared to 2 and none in the tramadol 2 and 4 mg/kg group respectively. Serum cortisol in the pethidine group was higher than baseline at post extubation 1, 3 and 6 hours, and were higher than both tramadol groups at 6 hours. Results of this study suggest that pre-operative tramadol may be more effective than pethidine to confer analgesia for up to 6 hours post-OHE.

CONCLUSION

In conclusion, tramadol has been found to be effective in managing acute post-operative pain in both dogs and cats, when administered pre-emptively at doses between 2 – 4 mg/kg via parenteral routes in procedures such as ovariohysterectomy and castration. A study that utilised the SC route and repeat dosing every 8 hours following maxillectomy or mandibulectomy in dogs reported adequate pain control. Thus, adequate analgesia can be promoted with increase doses or repeated dosing following orthopaedic procedures. Repeated oral doses of tramadol as the sole analgesic were found to be less effective in dogs undergoing TPLO. Combinations of tramadol and a non-steroidal anti-inflammatory drug were found to provide better post-operative analgesia in both dogs and cats.

CONFLICT OF INTEREST

There is no conflict of interest between the authors in writing this review article.

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Reviews

WORLD ZOONOSES DAY AND SOME OF THE IMPORTANT ZOONOSES IN MALAYSIA

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SUMMARY

The paper discussed briefly impact of zoonoses, the need for World Zoonoses Day and five of the important zoonotic diseases under surveillance in Malaysia, namely leptospirosis, brucellosis, Japanese encephalitis, malaria and rabies.

Keywords: Zoonoses, leptospirosis, brucellosis, Japanese encephalitis, rabies, malaria

INTRODUCTION

The word 'zoonoses' is combined from two Greek words—zoon (animal) and noses (an ailment). The word was introduced by Rudolf Virchow in 1880 to include collectively the diseases shared in nature by man and animals. In 1959, the World Health Organization (WHO) officially recognised the word and defined zoonoses as diseases and infections that are naturally transmitted between vertebrate animals and humans. There are approximately 1400 human pathogens, of which about 800 species are zoonotic. Reportedly, there are about 180 – 200 zoonotic diseases, which are known to exist (TWC India Edit Team, 2020; Rantsios, 2016).

Zoonotic diseases pose not only as major public health threat but also of economic importance and hurdles to food security and food safety especially with regards to food of animal origin. The World Bank estimated from six major zoonotic disease epidemics between years 1997 to 2009, incidences of Nipah Virus infection (Malaysia), West Nile Fever (USA), Severe Acute Respiratory Syndrome (SARS) (Asia, Canada and others), Highly Pathogenic Avian Influenza (HPAI) (Asia, Europe), Bovine Spongiform Encephalopathy (BSE) (US, UK) and Rift Valley Fever (Tanzania, Kenya, Somalia) an economic loss of more than US$80 billion in total. This was due to high morbidity and mortality rates in humans and animals, disruption of regional and global trade, strains on the national and global public health resources and substantial economic costs including medical / healthcare expenses, productivity loss and control measures. There are several pathways in which humans can be exposed to zoonotic diseases, often through direct contact with animals including being bitten or scratched, indirectly transmitted through food of animal origin, vector-borne, contaminated environment and via airborne transmission such as due to inhalation of microbial aerosol.

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Rantsios (2016) gave a very comprehensive overview on zoonoses which include the environmental aspects which show the complexities of the ecosystems in which animals and humans co-exist that may cause diseases to occur; the One Health approach to tackle zoonotic diseases through cross-sectorals and multidisciplinary cooperation; a concise list of zoonoses; in a list of the most important zoonoses in terms of human health impact, livestock impact, amenability to agricultural intervention, severity of diseases and emergence, the top three are gastrointestinal zoonoses, leptospirosis and cysticercosis; and in a list of zoonoses emergence linked to agricultural intensification and environmental change, among the examples mentioned were Nipah viral infections in Malaysia, Bangladesh and India.

Today, of the approximate 180 emerging or reemerging pathogens reported in the past three decades, 75% of these pathogen are known to be zoonotic. World Zoonoses Day 2020 came amidst an unprecedented global health crisis which WHO had declared as a pandemic due to the deadly novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2 or COVID-19) - an outbreak which started in Wuhan, China in December 2019 and was thought to have originated from a market that sold wild animals. Populations worldwide must constantly be made aware, educated and reminded that animals and environment should be looked after with proper management and care due to their important roles in the emergence and re-emergence of zoonoses.

World Zoonoses Day

World Zoonoses Day is held every year to commemorate the work of a French biologist, Louis Pasteur who on 6th of July 1885 successfully administered the first vaccine against rabies, a zoonotic disease. Up till today, rabies remains and is regarded as one of the most deadly zoonotic diseases, however only preventable with vaccination in disease control. World Zoonoses Day is also marked to raise awareness about the potential risk of zoonotic diseases, such as Ebola, Avian influenza, SARS, Middle East respiratory syndrome coronavirus (MERS-CoV) and many other zoonotic diseases. To this very day (1st

November 2020), COVID-19 has spread to over 215 countries affecting 48 million people directly causing 1.2 million deaths. The three countries seriously affected are USA, India and Brazil. In Malaysia, to date about 34,000 people were diagnosed with 263 reported deaths.

A report on "Preventing the Next Pandemic: Zoonotic diseases and how to break the chain of transmission", a joint effort by the United Nations Environment Programme (UNEP) and the International Livestock Research Institute (ILRI), was launched on World Zoonoses Day 2020. From what the world had experienced with regard to several epidemics and pandemics in the last two decades (as those mentioned above and with quite recent ones included Zika (2015–2016), Ebola (2014– 2015), MERS-CoV (2012), Swine Flu (2009) saw high morbidities and mortalities among animals and human and as a consequence had disrupted the economy of the affected countries. This report warned that further outbreaks will emerge and that all countries should undertake active and effective measures to prevent other zoonotic diseases and future pandemics. The report identified seven trends as drivers of the increasing emergence of zoonotic diseases, namely increased demand for animal protein; a rise in intense and unsustainable farming; the increased use and exploitation of wildlife; the climate crisis, travel and transportation, changes in food supply chain and unsustainable utilization of natural resources

It was well said by UNEP Executive Director Inger Andersen and most would agree that "The science is clear that if we keep exploiting wildlife and destroying our ecosystems, then we can expect to see a steady stream of these diseases jumping from animals to humans in the years ahead. Pandemics are devastating to our lives and our economies, and as we have seen over the past months, it is the poorest and the most vulnerable who suffer the most. To prevent future outbreaks, we must become much more deliberate about protecting our natural environment". Experts agreed that in order to control such disease outbreaks, it is imperative to minimise the overlap of boundaries between humans and wildlife. It is regarded that at present time, the main reason for the spread of zoonotic diseases to be due to increase in human contact with wildlife. Wild animals and their habitats today are threatened due to ongoing fragmentation, deforestation, intensive agricultural practices, construction, mining and other natural factors affecting the climate and environment as well as human factors resulting in loss of wild animal habitats. In such events and without much choice, these wild animals tend to move to human-dominated landscapes and indirectly had caused diseases to emerge, increase and spread. Moreover, relentless wildlife trade is also considered to be a major reason behind the extinction of ecologically valuable species. According to One Health experts, a healthy ecosystem can help protect against the emergence, re-emergence and as well as spread of several diseases. Therefore the protection of animal landscape may play an essential role in the prevention and control of zoonotic infections in nature. It can be grievingly seen that zoonotic epidemics and pandemics both have very devastating effects. Thus, these horrifying events must be remembered as a lesson and must be acted upon to stop further outbreaks in the world. Prevention of emergence of zoonotic diseases can only be achieved by protecting the animals and their natural habitats with the right governance, policies, implementation, monitoring and enforcement.

In Malaysia, a press statement on 6th July 2020 from Ministry of Health Malaysia not only updated on COVID-19 situation but also gave a brief report on the current status on six of the many zoonotic diseases. According to the same report from Director General of Health Malaysia, "The practice of good personal hygiene and the implementation of the Movement Control Order (MCO) in our country have in fact contributed to the reduction of zoonotic diseases such as Leptospirosis, Rabies, Brucellosis, Japanese Encephalitis, Q Fever and Malaria *Knowlesi*" as shown in Table 1 below. The report also mentioned some very brief history or write-up on the aforementioned diseases and included Nipah viral infection, SARS, Avian influenza and salmonellosis.

Some Important Zoonoses in Malaysia

Following the Nipah viral disease outbreak in 1999, Inter- Ministerial Committee on the Control of Zoonotic Diseases was established and co-chaired by Director General of Health, Ministry of Health and Director General of Department of Veterinary Services. In 2003, 15 zoonoses – namely Nipah viral infection, influenza (especially HPAI and other Influenza – human, equine, swine, avian), rabies, brucellosis, tuberculosis, BSE / vCJD, Japanese encephalitis, anthrax, leptospirosis, toxoplasmosis, Rift Valley fever, Q fever, filariasis, Yellow fever and three (3) zoonotic pathogens – *Salmonella enteritidis* and *S. typhimurium*, Vancomycin Resistant Enterococci (VRE) and Hanta virus are being placed under surveillance in Malaysia. In recent years, Ebola HF, MERS-CoV and Malaria *Knowlesi* are included in the list and recently COVID-19 is believed to have been added.

Table 1. Number of cases of six of zoonotic diseases in Malaysia, 2016 – June 2020

Diseases	2016	2017	2018	2019	June 2020
Leptospirosis	5,285	4,365	5,056	5,217	1,484
Rabies	0	6	10	6	2
Brucellosis	26	42	40	10	0
JE	49	22	28	36	7
Q Fever	6	6	1	0	0
Malaria *Knowlesi*	1600	3614	4131	3222	1156

(Source : Ministry of Health, 2020)

Here, in this series, we would like to present some write-up on episode and / or update the status on five of the diseases.

Leptospirosis

Leptospirosis is endemic in the country, in both human and animal populations as well as in the environment. It was reported that the wide range of domestic and wild animals as reservoirs, the humid and moist environment as well as the abundance of forest settings are suitable for proliferation of the organisms. Moreover, the indiscriminate disposal of garbage and food wastes not only in residential areas, around town / cities as well recreational areas has caused an increased in the presence of rats which have been mostly reported to be the sources of leptospires. Of the 250 known *Leptospira* serovars, about 38 serovars had been identified in Malaysia. Apart from rats, a number of studies had also reported incidence of leptospirosis in cattle, pigs and to a lesser extent sheep, goats and dogs as reservoirs of *Leptosira Icterohaemorrhagiae, Hardjo, Bratislava, Pomona* and *Canicola*, respectively. The infected animals may become carriers with the organisms present in the renal tubules for periods of days, months to years and shed the leptospires in urine which then directly contaminate the environment. Human leptospirosis often resulted from direct or indirect contact with contaminated urine, water, mud or soil and with infected animals (Garba *et al.*, 2017; Thayaparan *et al.*, 2013, Alashraf *et al.*, 2019; Goh *et al.*, 2019).

One outbreak that attracted international attention was reported in year 2000 where 68 teams which consisted of four athlete members per team competed in an Eco-Challenge in Borneo (Sabah) at Segama River. The events held had many water activities such as kayaking, swimming and spelunking. Of 304 athletes that were contacted (189 from the United States and 26 other countries), 80 or 42% athletes met the case definition of the investigation with 29 or 36% case-patients were hospitalized and fortunately no death recorded. Investigations also found the swallowing of water from the Segama River as an exposure risk factor (Sejvar *et al.*, 2012).

There were two outbreaks that caught national attention at the time. In October 1999, 46 males, aged between 8 to 19 years were admitted to Beaufort Hospital (100 km from Kota Kinabalu, Sabah) after swimming in a creek near an oil palm plantation in Kampung (Kg) Kebatu, Beaufort. Unfortunately one death was recorded. The water body was suspected to have been contaminated by leptospires due to stagnation and flooding resulted from heavy rainfall in the area earlier to the episode. In June 2010, an outbreak of melioidosis co-infection with leptospirosis involved 153 individuals who took part in a rescue mission to find the body of a young man who has allegedly drown in Lubuk Yu recreational forest with waterfall and stream (about 130km from Kuantan) in Pahang. Among the 10 confirmed melioidosis cases, four were co-infected with leptospirosis. Unfortunately, eight people died, consisted of seven volunteer villagers and one professional rescuer with overall case fatality at 70% which may have been aggravated by the fact that all the positive cases had diabetes mellitus. Other significant finding in this study was 100% case fatality rate in the volunteer villagers compared to 33.3% in professional rescuers. Thus, the report suggested that non-professional rescue workers should be discouraged from taking part in such rescue mission in future (Sapian *et al*., 2012; Koay *et al*., 2004).

Brucellosis

Ten species have been recognized within the genus *Brucella*. However, there were 6 "classical" species based mainly on differences in pathogenicity and host preference, namely *B. abortus, B. melitensis, B. suis B. neotomae, B. ovis* and *B. canis.* The occurrences of brucellosis in the livestock population locally have been reported for many decades but with low prevalence when compared to other countries in Asia. Brucellosis is an economically important disease affecting animal productivity, causing abortion, infertility, decreased milk production and costs of replacement animals which directly contribute to economic losses. It was reported there was a significant surge in the sero-prevalence of brucellosis in goats, mainly due to *B. melitensis* at 0.91% and the trend had continued to increase in recent years. Similarly, brucellosis in cattle caused by *B. abortus* was reported to be widespread among herds in Peninsular Malaysia with prevalence of 21.8%. In human, a prevalence of 5.8% have been reported in hospital patients with history of association with animals. It is also very interesting to note a prevalence of 14.2% was reported among veterinarians, indicating that veterinarians and farmers were at high risk of contracting the disease due to constant close contact with animals (Yahaya *et al*., 2019)..

Thus far, the largest outbreak of brucellosis in the country was reported in Penang. A goat farm owner, with more than 300 goats were infected. The farmer himself had consumed the raw goat milk produced which he had also sold to the public. From the hospital records, 83 patients were diagnosed with brucellosis which consisted of 79 patients were linked to the consumption of the raw milk, 2 farm workers and 4 laboratory staff who were presumed to have contracted the disease through handling of blood samples (Kar *et al*., 2015).

Brucellosis has been reported as an important cause of laboratory outbreaks and may account for up to 2% of laboratory-associated infections with attack rate around 30%–100%, depending on the inoculum involved, the physical location of the workers, and the source at the moment of the exposure. In 2011, a large exposure to *B. melitensis* in a university diagnostic laboratory in Kuala Lumpur was reported; of the 51 staff, 27 of them were considered to have high-risk exposure as they handled open cultures (n=12) and were within 1.5 m from the open cultures (n=15). These individuals were offered post-exposure prophylaxis and fortunately no clinical brucellosis was detected. In another incident in 2014, a clinical brucellosis was reported in a research assistant who worked in a veterinary microbiology laboratory in Serdang. The individual was confirmed based on the

presence of high level of antibody titer against *B. melitensis.* Aerosolisation is the primary mechanism of transmission in laboratory setting. Therefore, it is recommended that the organisms should be handled according to biosafety level 3 (BSL 3) precautions (Sam *et al*., 2012; Hartady *et al*., 2014).

Japanese Encephalitis

Japanese encephalitis (JE) is a vector-borne zoonotic disease caused by the Japanese encephalitis virus (JEV), spread by the bite of *Culex* spp. mainly *Culex tritaeniorhynchus*. JEV belongs to the genus Flavivirus and to date, is classified into five genotypes which have been isolated in the Malaysia-Indonesia regions. Pigs and wading or ardeidae birds (such as herons and egrets) are the principal amplifying hosts producing high viraemia that infect the mosquito vectors that play an important roles in the maintenance and transmission of the disease. A vast variety of animals can be infected with JEV includes horses, dogs, chickens, ducks, cattle, cats, bats, snakes, frogs, sheep, goats, wild boars, monkeys, raccoons, water buffalos, and birds. JE is not considered a serious public health problem in Malaysia, except for Sarawak with human mortality rate of 9% (Kumar *et al*., 2018a).

Since 1942, JE cases and outbreaks have been reported not only in humans but also animals. A recent study in the country reported seroprevalence data in dogs (80%), pigs (44.4%), cattle (32.2%), birds (28.9%), cats (14.4%) and monkeys (14.3%); previous serological studies reported not only in these animals but also in horses, goats, sheep, wild boars and in mosquitoes (Kumar *et al*., 2018b). One of the notably known outbreak of JE was in 1998 – 1999 in Perak and Negeri Sembilan that occurred along with Nipah viral infection outbreak. The occurrence of encephalitis in significant numbers of death in vaccinated pigs and that only pig farmers and workers were affected suggested that another agent was responsible which was then identified as a new emerging Nipah viral infection. There were 157 cases and 58 deaths with JE confirmed to be the cause of 18 of the reported deaths. It has been suggested that since these outbreaks or cases that occurred in Malaysia with no pig farms or other amplifying hosts in those areas, the diversion could it be that bats may play a role (Kumar *et al*., 2018a). A number of studies had indicated that bats may be involved in transmission of JEV, however so far to our knowledge there is still no study of JEV among bats in Malaysia.

Malaria

Approximately 250 species of *Plasmodium* have been believed to parasitise animals, birds and reptiles and of these, about more than 30 species have been reported in non-human primates, including apes, gibbons and monkeys. Four *Plasmodium* species that considered humans as natural hosts and can be transmitted between humans by *Anopheles* mosquitoes, namely *Plasmodium falciparum, P. malariae, P. ovale* and *P. vivax.*. Human infections with simian malaria parasites were initially thought to rarely infect humans until *P. knowlesi*, a common parasite identified mainly in macaque monkeys has now been discovered to cause a surge of incidence of human malaria in Sarawak (Ramasamy, 2014; Raja *et al*., 2020).

Further investigations and studies using molecular methods, revealed interesting discoveries over the years namely – i) the routine microscopy examination of blood films misidentified *P. knowlesi* as *P. malariae* and that *P. knowlesi* malaria in humans is actually very widespread not only in Sarawak and Sabah, but alarmingly also in Peninsular Malaysia; ii) *P. knowlesi* was reported at 96% of all malaria cases in one hospital in the peninsular; iii) mixed infections of *P. knowlesi* and human malaria parasites in patients in Peninsular Malaysia, Sarawak and Sabah and as well as *P. knowlesi* and *P. cynomolgi* co-infection in patients in Sarawak were observed; iv) erroneous microscopic identification of *P. cynomolgi* as *P. vivax*; v) macaques in Sarawak found to harbour six malaria parasites, namely *P. inui, P. knowlesi, P. cynomolgi, P. coatneyi, P. fieldi* and *P. simiovale*; and vi) studies on experimental and accidental infections has proven that apart from *P. knowlesi* and *P. cynomolgi, P. inui* also has zoonotic capability. These important findings have directly contributed to a better understanding of the disease, treatment and strategies required to be implemented for disease control (Ramasamy, 2014; Raja *et al.,* 2020).

Rabies

Rabies was first reported to occur in Malaysia since 1884, however records of human cases were found in the beginning of year 1924. Majority of the cases were found in states bordering Thailand and these major outbreaks had called for National Rabies Control Programme to intervene and control. Malaysia was declared rabies-free in 1954 and an immune belt was established in the states bordering Malaysia – Thailand. However, sporadic cases in humans and dogs still occurred and with organised efforts to control the disease, the country was then declared as rabies-free again in 1999. Outbreaks and sporadic cases still occurred and with more diligent actions, the country was declared rabies-free in 2013. Sadly, despite all the efforts taken to control, the rabies-free status of Malaysia was shattered in year 2015 due to outbreaks that occurred in Perlis, Penang and Kedah, and this time only dog cases were reported with no human case. Again in 2017 and 2018, there were reported episodes of rabies in Perak and Perlis.

In summary, the occurrences of rabies were in Peninsular Malaysia all these years. Only in July 2017, rabies outbreak was also reported in Sarawak. Despite efforts to control the disease, by December 2018 there were 332 confirmed rabies cases in dogs and 16 cases in humans with 15 deaths and one survived with severe neurological complications. As of 2019 to November 2020, there were 14 more human cases with a total of 30 cases to date including 28 deaths. It has been suggested and agreed upon to establish an immune belt at Sarawak – Kalimantan (Indonesia) border, about 650 km in length. To control the outbreak, much awareness and knowledge

has been instilled among the affected communities on how to first clean their own wound in any incidence of dog bites that would occurred and then report at the local clinic/hospital. In dogs, a massive rabies vaccination for dogs had been organized with firstly on all the pet dogs and then stray dogs of the local communities (Navanithakumar *et al*., 2019; Muthu, 2018)

World Rabies Day is held annually on September 28. It is the anniversary of the death of Louis Pasteur, who developed the first rabies vaccine and laid the foundations of rabies prevention. The theme for the WRD 2020 is **"**End Rabies: Collaborate, Vaccinate" and it called upon all stakeholders to join hands and collaborate in efforts to eliminate dog-mediated human rabies by 2030 ("Zero-by-30"). The theme also reiterates the need to invest and focus on dog rabies vaccination to achieve the global goal. In Malaysia, the Department of Veterinary Services together with the Faculty of Veterinary Medicine in Universiti Putra Malaysia organised a seminar presenting a number of important issues related to rabies by relevant government agencies (World Rabies Day 2020).

CONLUSION (Epilogue)

Human health and well-being and animal health and welfare are strongly interlinked and both influence and are impacted by healthy environment. Hence there is a need for effective and sustained cooperation between various agencies, professions and disciplines, in both public and private sectors in handling zoonotic diseases epidemics. This One Health approach will certainly ensure in achieving enduring health for human, animals and the environment.

CONFLICT OF INTEREST

There is no conflict of interest between the authors in writing this review article.

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Journal Articles

PREVALENCE OF DEMODICOSIS IN DOGS AND ITS ASSOCIATED RISK FACTORS IN A SMALL ANIMAL HOSPITAL IN PENANG, MALAYSIA

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SUMMARY

A study was conducted to investigate the prevalence of canine demodicosis and its associated risk factors in dogs that was presented to a veterinary hospital in Penang from the period of January 2016 to December 2018. Risk factors such as breed, age, sex, management and time of occurrence were investigated. A total of 85 positive cases were reviewed from the total sample of 5810 dogs. The overall prevalence of demodicosis recorded in the period of three years was 1.46% (n=85). Pure breed dogs were highly predisposed to demodicosis followed by the mixed breeds (n=27, 32%) and mongrels (n=2, 2%). Male dogs (n= 47, 52%) were affected than female dogs (n=42, 47%). Older dogs (n=31, 37%) were more affected comparatively followed by the puppies (n=24, 28%) and then the adult age group (n=17, 20%). Most dogs that were affected with demodicosis were indoors (n=82, 96%), followed by stray dogs (n=3, 4%), and cases were found to be higher during the months of March and May. The percentage of demodicosis was highest during the year of 2018, followed by the year 2016 and 2017 respectively. This study provides baseline information of incidence of demodicosis using patient records but similar studies must be conducted in other veterinary clinics/hospitals in order to get a better estimate of the prevalence of canine demodicosis.

Keywords: demodicosis, dog, prevalence, Demodex sp, ectoparasites

INTRODUCTION

Demodicosis is one of the major skin disease of dogs caused by mites of various *Demodex species* (Shrestha *et al.*, 2015). There are three species of Demodex mites that are found in dogs namely *Demodex canis*, the long-bodied *Demodex injai* and the *Demodex cornei*, short bodied mite (Mueller, 2004).

Demodex canis is an acarine parasite which resides as a normal inhabitant at the hair follicles and sebaceous glands of the dog's skin (Solanki, 2005). There are two forms of demodicosis: localized or generalised form with the clinical manifestation of pustular and squamous type of skin lesions (Shrestha *et al.*, 2015). Demodicosis is considered to be localised if there are only four lesions and below with a diameter up to 2.5 cm and generalised when there are more than four lesions that occupy 50% of the body surface (Mueller *et al*., 2011). An altered immune response in young dogs and immunosuppressive treatment or immunosuppression in adult dogs triggers hyperproliferation of mites that will lead to the development of clinical signs. Young animals which suffer from malnutrition and endoparasitisicm will cause immunosuppression that promotes mite proliferation. In older animals, disease such as neoplasm, hypothyroidism which suppress the immune system of the dog can sufficiently lead to mite's proliferation (Mueller *et al*., 2011).

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Hence, it is vital to comprehend the predisposing factors and its associated risk factors to tackle and prevent the disease. There are limited information and study on the prevalence rate of demodicosis reported in dogs in Malaysia. Therefore, this study was undertaken due to lack of data on the incidence of demodicosis associated with risk factors in dogs. The aim of this retrospective study was to analyse the prevalence and its risk factors associated with breeds, age, sex, management and the time period of occurrence that were presented to the veterinary hospital between January 2016 and December 2018. Thus, the results from this study will be useful for the clinicians to understand the associated risk factor contributing towards this disease.

MATERIALS AND METHODS

Patient's record from the veterinary hospital in Penang were reviewed retrospectively. Data from January 2016 to December 2018 were collected to analyse the prevalence rate of canine demodicosis. Cases that were positive for demodex mites from the deep skin scrapings were included in the study. The details of dog patients that were confirmed with demodicosis were collected based on the animal's information (breed, age, sex, size, weight) and time of occurrence (monthly and yearly) were recorded.

The data were then analysed for the prevalence of demodicosis and the frequency distribution of risk factors were calculated using Microsoft Office Excel 2010. The values were represented in frequencies and percentages. The distribution of demodicosis in dogs was divided into groups in accordance to the risk factors. Outcomes of each aspect were evaluated and were expressed as a percentage of the total number of outcomes from all aspect by using tables and charts.

RESULTS

Out of the total number of 5810 dogs that were presented from January 2016 to December 2018 only 85 (1.46%) cases were positive for canine demodicosis.

Breeds were categorised into 3 groups: pure breed, mix breed and mongrel. Table 1 shows the number and percentage of demodicosis according to breed classifications. The percentage of pure breed affected was the highest 66% (n=56) followed by mix breed 32% (n=27) and mongrel 2% (n=2). This shows that the pure breed had a higher predilection comparatively than other two groups.

Table 1. Percentage of demodicosis in dogs according to breed, sex, neutering status, age, weight and management

Risk factors	Number	Percentage (%)
Breed		
Pure	56	66
Mix	27	32
Mongrel	2	2
Sex		
Male	46	54
Female	39	46
Neutering status		
Neutered	11	13
Intact	74	87
Age		
Puppy (up to 2 years)	24	28
Adult (3-8 years)	17	20
Older (> 8 years)	31	37
Not known	13	15
Weight (kg)		
0-10	41	48
11-20	26	31
21-30	13	15
31-40	5	6
Management		
Indoor	82	96
Outdoor	3	4

As for sex, male dogs (n=47, 52%) were more affected than females (n=42, 47%) Table 1. Apart from this, intact dogs (n=74, 85%) were more predisposed to demodicosis, than neutered dogs (n=11, 15%).

This study also revealed that older dogs that are more than 8 years old (Table 1) were more predisposed to demodicosis (n=31, 37%), followed by puppies up to 2 years old (n=24, 28%) and adult dogs that ranges from 3 years to 8 years old (n=1, 20%). There are 13 cases with no information of the age. Adding to this, percentage of demodicosis according to weight showed that dogs between 1-10 kg (n=41, 48%) group are the most accounted with demodicosis followed by the weight group of 11-20 kg (n=26, 31%), 21-30 kg (n=13, 15%) and 31-40 kg (n=5, 6%) respectively (Table 1). Dogs that are kept indoor are most affected (n=82, 96%), (Table 1), compared to outdoor dogs (n=3, 4%)

This study documented the pattern of distribution throughout the 3 years, the number of cases was highest in 2018 with total positive cases of 33 (39%), followed by 2016 with 28 cases (33%) reported and the least number of cases was in 2017 where only 24 cases (28%) were recorded (Figure 1). The total number of cases were highest in the month of March (13%) and May (13%) throughout the three years. The least number of cases were recorded in the month of June (2%) (Figure 2).

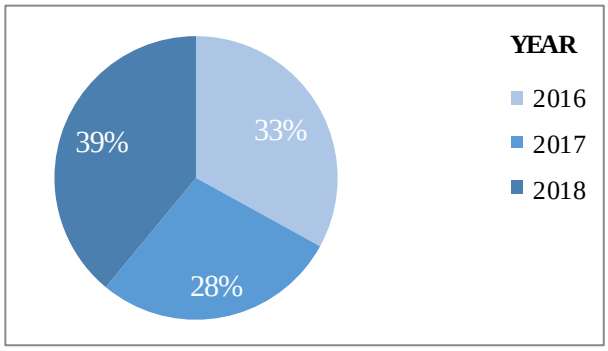

Figure 1. The total percentage of demodicosis in the period of three years

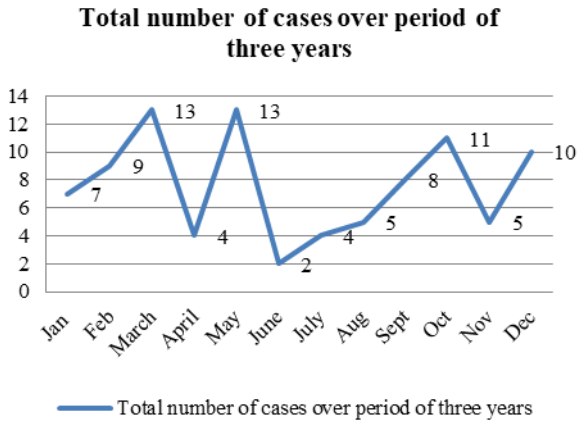

Figure 2. The total number of cases over period of three years

DISCUSSION

A total of 5810 dogs were registered at the hospital from January 2016 to December 2018 due to various reasons. Out of the total number of dogs, only 85 cases were identified positive for demodicosis. The incidence for the positive demodicosis cases that has been recorded was 1.46% (85/5810). This could be due to low cases of demodicosis that has been encountered in the hospital. The accuracy of prevalence rate can be achieved better if the sample size is bigger.

Even though *Demodex canis* is one of the commensals that lives under the skin of the dog, several underlying factors such as immunosuppression and breach of skin barrier can lead to the manifestation of the disease. This correlates with statement by Gunaseelan (2011) where factors such as environmental influences

and resistance level of the animals can also contribute towards the varying degree of prevalence.

The prevalence rate in this study is slightly higher than the study conducted by Bowden (2017), where the prevalence rate recorded was 0.37% and slightly lower comparatively than the study by Chee (2008), using the sample of stray dogs where *Demodex canis* was detected around 4.9% in Gwang Ju city, Republic of Korea. All these study shares the same method for sample collection where deep skin scrapings was used. Nevertheless, the variance of prevalence rate can be due to a number of epidemiological factors, such as weather, seasonal variations, geographical location, and breed differences.

The current study also revealed that pure breeds recorded the highest percentage of demodicosis 66% (n=56) which suggest that purebreds are more susceptible to demodicosis. Similar results were shown in a study by Solanki (2007) where pure breeds have higher affinity towards demodicosis. The susceptibility of demodicosis in purebred dogs could also be due to the autosomal recessive hypothesis that leads to immune dysfunction (Gortel, 2006). Apart from this, some herding breeds have a genetic defect resulting from a mutation in the multi-drug resistance gene (MDR1 gene), which makes these breed more susceptible to adverse effect of the macrocylic lactones drugs such as ivermectin, toxicity.(Mealey, 2016).

Mix breed were the most common breed presented (n=27) followed by Shih Tzu (n=16) for canine demodicosis. This correlates with the study by Bowden (2017) where mixed breed dogs (n=26/139; 18.7%) were the most affected by demodicosis, followed by the Shih Tzu (n=12/139; 8.6%), and other breeds. This is probably due to the preferences of mix breeds dogs as they are more affordable than pure breeds.

In this study, only two positive cases were recorded by the mongrel breed where the prevalence rate was 2.4% which shares a slight close proximity from the studies conducted by Kumar (2018) in Bihar, India where the detection rate was 3.16% in mongrel. This could be due to the preference of people on choosing the breed of choice to rear as a pet. Mongrel could be a less preferred breed of choice as people nowadays are fonder towards small sized pure breed of dogs as a companion animal.

Another interesting finding in this study is that the prevalence of demodicosis was more frequent in male dogs than in female dogs. This finding is in consistent with the findings by several studies (Sharma *et al*., 2018; Solanki *et al*., 2007; Kumar *et al*., 2006). In contrast, Islam (2013) and Rodriguez-Vivas (2003) stated the prevalence was higher in female dogs. There are many attributing factors that can lead to this such as, innate resistance, weather, locations, hormonal level and the age of the animals in the study (Shrestha *et al.*, 2015; Chee *et al.*, 2008).

Age wise revealed that older dogs that are more than 8 years old were most susceptible to demodicosis compared to young and adult dogs. Findings by Kumar (2018) stated that older dogs with weakened immune system due to malnutrition and health debilitation can aggravate the condition. Another studies found a contrasting result, where puppies up to one year had the highest susceptibility to demodicosis (Berian *et al*., 2018; Sharma *et al*., 2018; Solanki *et al*., 2007). This could be due to inheritance of the condition from the parent or due to the undeveloped immune status of the dog that could easily predispose them to demodicosis. However, due to lack of complete history of the dogs, we can only postulate the condition. Therefore, detailed history, physical examination and complete diagnostic work is important in order to identify the underlying cause of the disease.

Another attributing factor that can attribute to demodicosis is the weight of the dogs. In this study, dogs with the weight distribution between 1-10 kg (n=41, 48%) were the most affected with demodicosis. Not only that, most dogs in this study that falls in this range of weight distribution are from small sized breed.

Ninety six percent of dogs that are affected by demodicosis in this study were kept indoor and could reflect the urbanised demographic where the practice is located. The current study only focused on dogs that were brought to this hospital and thus the results obtained could be side-lined towards housed kept dogs.

During the study period, prevalence of demodicosis was highest during the year 2018, followed by 2016 and 2017 respectively. The rate of infections during this period showed two peaks that is in March and May. The least number of cases were recorded in the month of July. These findings were similar with the other studies (Gunaseelan *et al*., 2011; Solanki *et al*., 2007; Anish *et al*., 2018) where the demodex infestation was at peak in March and during summer. High temperature and humidity during this time is a favourable factor for the development and prevalence of demodicosis.

CONCLUSION

Canine demodicosis is a common skin problem in dogs with a low prevalence rate of 1.46% recorded from January 2016 to December 2018 at a veterinary hospital in Penang. Most cases were encountered during March and May. Age and breed of dogs were highly associated with demodicosis, but both sexes can be equally affected. Geriatric dogs and pure breed dogs were more predisposed towards the disease even though the mixed breeds showed the most number of cases recorded in this study. This is just a preliminary study and focused only in one animal hospital in Malaysia. Similar study needs to be conducted in other areas within Malaysia to get a better conclusion.

CONFLICT OF INTEREST

None of the authors of this paper has financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Case Reports

ACUTE DYSPNOEA OF DIFFERENT AETIOLOGIES IN TWO MALTESE DOGS

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SUMMARY

Two Maltese dogs showing acute dyspnoea were presented for a cardiology examination after an initial stabilisation by the respective primary veterinarians. The chest radiography and echocardiography were interpreted cautiously since the dogs were given multiple, high-doses of furosemide prior to the examination. Left-sided congestive heart failure due to canine myxomatous mitral valve disease was diagnosed as the cause of dyspnoea in one dog, while the other dog had severe pulmonary hypertension unrelated to the left heart disease. Therefore, judicious use of diuretic therapy in dyspnoeic animals should be exercised.

Keywords: Dyspnea, myxomatous mitral valve disease, pulmonary hypertension, furosemide, pulmonary edema

INTRODUCTION

Dyspnoea refers to difficult, labored or painful breathing, and implies significant cardiac, respiratory, or other systemic problems. Differentials for dogs presented with acute dyspnoea include cardiogenic or non-cardiogenic pulmonary edema (PE), severe pneumonia, airway obstruction, pulmonary thromboembolism, pneumothorax (Smith et. al., 2008), and acute respiratory distress syndrome (DeClue et. al., 2007). Because successful management depends on the ability to identify the cause of dyspnoea to institute a specific therapy timely, this case repot describes the different clinical features of cardiac and respiratory causes of dyspnoea.

CASE REPORT

Case 1

A 10-year-old Maltese dog which weighed 4.2 kg. The dog was stabilised with furosemide (4 mg/kg) and oxygen supplementation by the primary veterinarian. Chest radiography was taken after it received 2 doses of furosemide, intramuscularly (IM) and intravenously (IV), respectively. The dog responded to the diuretic therapy and was able to rest comfortably on sternal position.

Upon the cardiology examination, the dog was alert and responsive but exhibited labored abdominal breathing and occasional exertional coughing. Auscultation revealed mild crackle of the left lungs, harsh bronchovesicular sound of the right lungs, tachycardia (heart rate 180 bpm), and left apical systolic murmur grade IV/VI.

The chest radiography was reviewed. On the right lateral view (Figure 1a), the cardiac silhouette was mildly enlarged [vertebral heart score (VHS): 10.5] whereas the left atrium was moderately enlarged [vertebral left atrium score (VLAS): 2.75]. Dorsal elevation of the trachea, compression of the main stem bronchi, and mixed lungs patterns were also observed. Further, the dorsoventral view (Figure 1b) showed dilated pulmonary veins and pulmonary alveolar infiltrates from the 5th to 7th

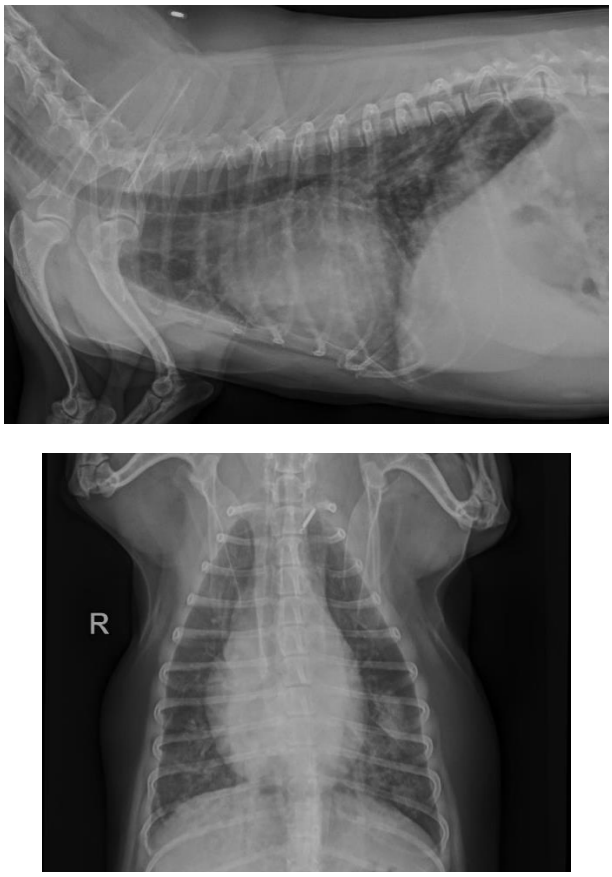

Figure 1. (a) (Top) Right lateral thoracic radiograph showing an enlarged cardiac silhouette and mixed lung patterns. (b) (Bottom) Dorsoventral thoracic radiograph showing bilateral pulmonary alveolar infiltrates which spanned from 7-9th intercostal spaces

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intercostal spaces, collectively suggestive of cardiogenic PE. Based on the clinical signs and radiographic findings, a diagnosis of left-sided congestive heart failure (CHF) was made.

Since the dog was already given two doses of the furosemide therapy, it was deemed beneficial to medicate it with pimobendan (Vetmedin, 0.30 mg/kg, PO) to further reduce preload and afterload, and pethidine (3 mg/kg, IM) as sedative and antitussive before echocardiography. Echocardiographic findings showed thickened anterior and posterior mitral valve (MV) leaflets which prolapsed into the left atrium (LA) during systole (Figure 2), severely dilated LA (LA/aorta (Ao): 2.1), mildly dilated left ventricular (LV) chamber [LV end-diastolic diameter (LVDD): 25.6 mm; LVDD normalized to body weight (LVDDN): 1.7]. Further, the LV exhibited hyperkinesia (FS: 54.7%) whereas the trans-mitral E wave velocity was measured 0.80 m/s. Besides, the lung ultrasonography showed multiple B lines of the left and right lungs (Figure 3), which strengthened the diagnosis of PE due to stage C myxomatous mitral valve disease (MMVD).

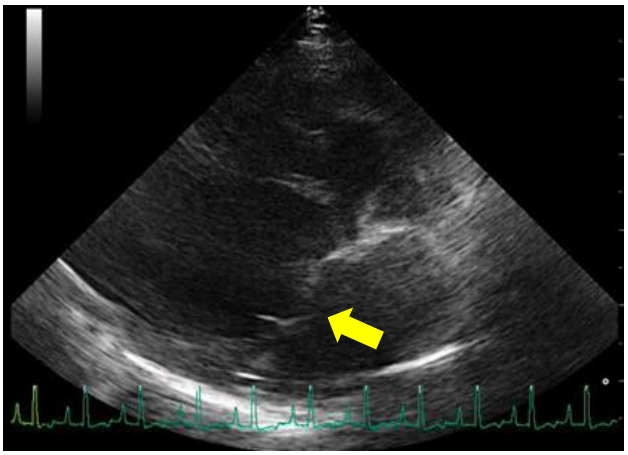

Figure 2. (Left) Right parasternal long-axis image showing dilated left atrium (LA) and left ventricle. The mitral valve leaflets (arrow) was thickened and prolapsed into the LA during systole.

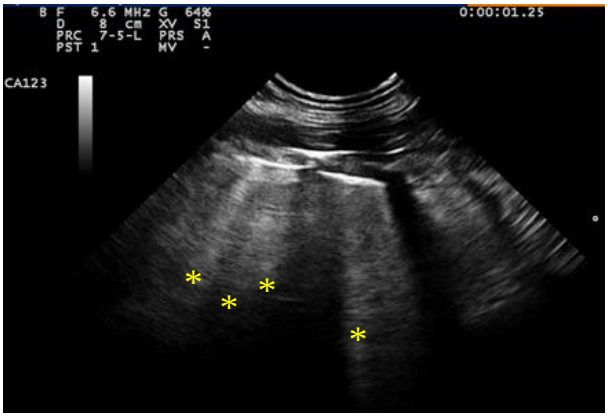

Figure 3. (Right) Lung ultrasound image showing multiple B-lines as indicated by asterisks.

In addition, electrocardiogram (Figure 4) showed sinus tachycardia (heart rate 181 bpm), mean electrical axis of 61°, P pulmonale (P wave amplitude: 0.6 mV), and an increased amplitude of the R wave (3.3 mV), suggesting left ventricular enlargement.

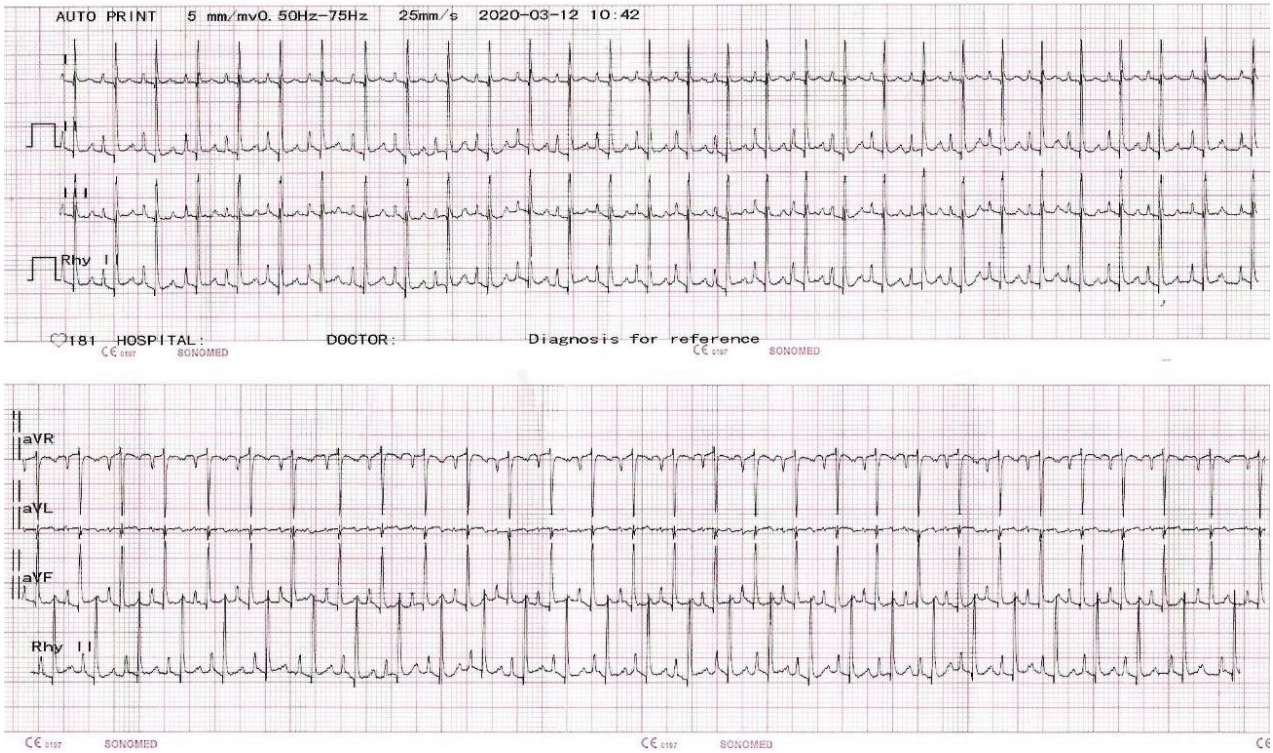

Figure 4. Electrocardiogram showing sinus tachycardia, P pulmonale, and increased R wave amplitude (sensitivity 5 mm/mV; paper speed 25 mm/s).

After the examination, recommendations were given to the veterinarian to manage the acute left-sided CHF by medications of furosemide (tapering to the lowest dose) and pimobendan. Monitoring of the renal parameters was also advised. Client education which included monitoring of sleeping respiratory rate was also suggested. A reevaluation was scheduled after 2 weeks to monitor the disease progression and review the medications.

Case 2

A 17-year-old Maltese dog which weighed 3.1 kg was treated by the primary veterinarian with furosemide (4 mg/kg), intravenously and oxygenation before the cardiology examination. Haematology showed mild anemia (Red blood cell: 5.23 x $10^{\wedge 12}$/L; Reference range: 5.65-8.87 x $10^{\wedge 12}$/L; Packed cell volume: 35.3%; Reference range: 37.3-61.7%; Haemoglobin: 11.8 g/dL; Reference range: 13.1-20.5 g/dL), left-shift leukocytosis (White blood cell: 23.54 x $10^{\wedge 9}$/L; Reference range: 5.05-16.75 x $10^{\wedge 9}$/L), neutrophilia (16.81 x $10^{\wedge 9}$/L; Reference range: 2.95-11.64 x $10^{\wedge 9}$/L), and monocytosis (2.05 x $10^{\wedge 9}$/L; Reference range: 0.16-1.12 x $10^{\wedge 9}$/L). As for biochemistry, only blood urea nitrogen (BUN: 4.58 mmol/L; Reference range: 2.5-9.6 mmol/L) and alkaline transaminase (ALT: 57.7 U/L; Reference range: 10-125 U/L) levels were determined, in which both were within the normal limits. Besides, the dog was tested negative for heartworm disease.

Upon physical examination, the dog was responsive but dyspnoeic. Auscultation revealed normal heart rate (120 bpm), left apical systolic murmur grade II/VI, right apical systolic murmur grade IV/VI, and lung crackles. Pulse oximetry showed hypoxaemia (SpO_2: 83%). The chest radiograph (Figure 5) showed mild cardiomegaly (VHS: 10.5) and heart apex elevation, indicating right heart enlargement. Bronchial patterns, and patchy pulmonary infiltrates suggesting PE were also observed.

Echocardiogram revealed moderately thickened MV leaflets, mild mitral regurgitations, normal LA (LA/Ao: 1.4) and LV (LVDD: 14.2 mm; LVDDN: 1.0) dimensions, and trans-mitral E velocity of 0.48 m/s. The above findings indicated a mild MMVD which was unlikely to cause of dyspnoea in the dog. Further, the slightly thickened LV wall (Interventricular end-diastolic wall thickness (IVSd): 7.6 mm; Posterior wall end-diastolic thickness (PWd): 6.4 mm) and the reduced LV diastolic dimension may be the combined results of pulmonary hypertension (PH) which resulted in left-sided unloading, and diuretic-induced hypovolaemia. In addition, the septum also showed paradoxical movement and systolic and diastolic flattening (Figure 6). Right atrial enlargement and tricuspid regurgitation (TR) with a velocity of 4.26 m/s were observed (Figure 7), so the systolic pulmonary arterial pressure was estimated at 72.5 mmHg. Besides, the pulmonary artery (PA) was dilated (PA/Ao: 1.4) and showed regurgitation velocity of 2.48 m/s (Figure 6) and type III systolic flow. Therefore, a diagnosis of highly probable PH was made. Because the dog had a history of chronic coughing, a group 3 PH secondary to respiratory disease was highly suspected, followed by pulmonary thromboembolism and less likely pneumonia.

Recommendations to medicate the dog with sildenafil citrate (Viagra 100 mg, 1/8 tab, 3 times daily), to continue oxygen supplementation, and to treat the respiratory disease were given. Also, the furosemide treatment was discontinued immediately. According to the veterinarian in-charge, the dog showed improvement

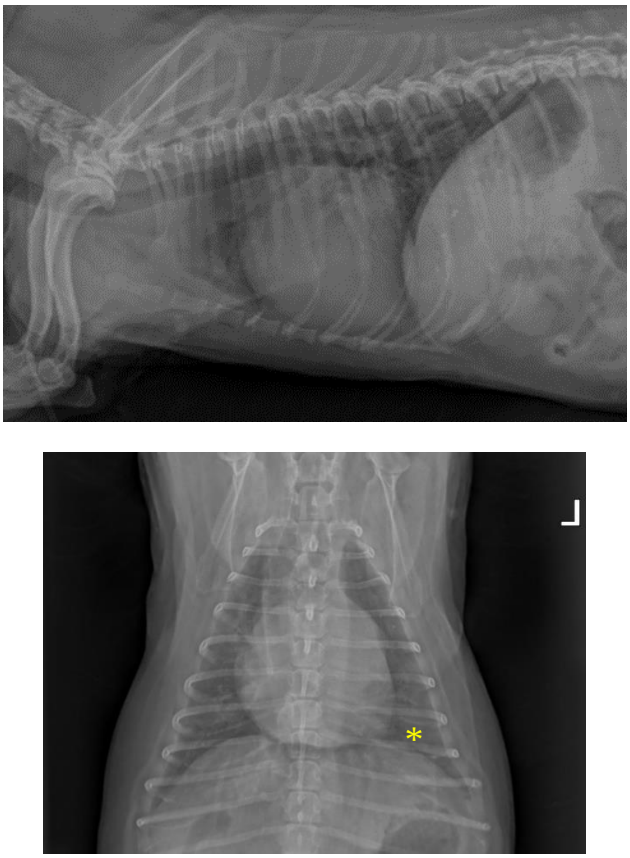

Figure 5. (a) (Top) Left lateral and (b) (Bottom) dorsoventral thoracic radiographs showing mild cardiomegaly and apex elevation (indicated by an asterisk), bronchial and patchy pulmonary alveolar infiltrates

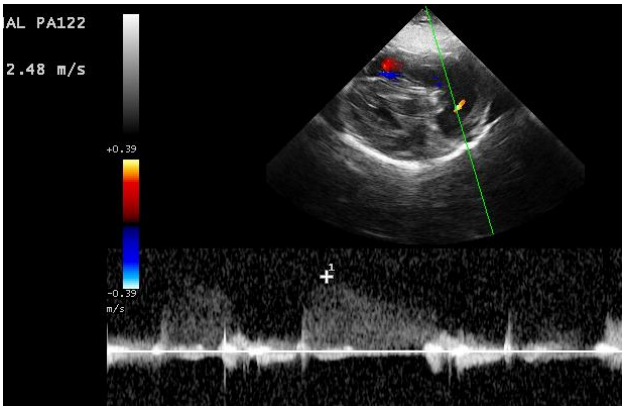

Figure 6. Right parasternal view of the right ventricular outflow tract showing pulmonic regurgitation with a velocity 2.48 m/s. Note the septal flattening, the thickened LV wall, and the reduced LV dimension.

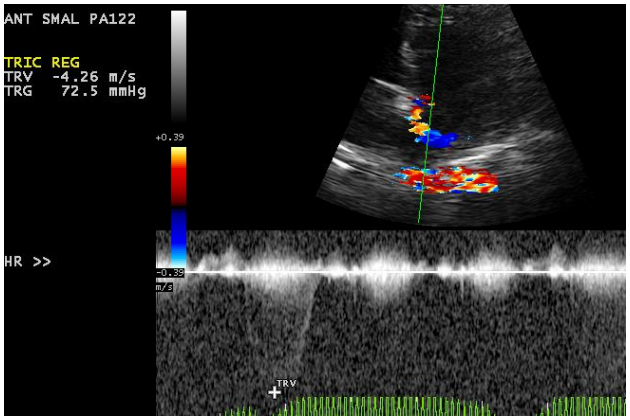

Figure 7. Doppler study showing tricuspid regurgitations which measured peak velocity of 4.26 m/s.

and started to eat and drink in the evening of the day admitted. However, on day 3, the dog was found dead about two hours after breakfast in the clinic.

DISCUSSION

The case report depicted two Maltese dogs which showed dyspnoea attributable to different etiologies: cardiogenic PE due to stage C MMVD in the case 1 and non-cardiogenic PE due to PH in the case 2. They were both treated symptomatically with furosemide and oxygenation prior to the cardiology examination. While the furosemide therapy is life-saving by reducing cardiogenic PE, it may be detrimental to non-cardiogenic PE by further decreasing the blood volume and hence cardiac output in a dog with compromised perfusion. Therefore, differentiating the cardiac from non-cardiac cause of dyspnea before administering furosemide to a dyspneic animal is indispensable.

Careful cardiac auscultation may provide an initial clue as murmur intensity in small-breed dogs with MMVD correlates to the disease severity (Ljungvall et. al., 2014). The CHF dog in the case 1 had a higher heart rate due to an upregulated sympathetic tone (Uechi et al., 2002) and stronger left apical systolic murmurs (grade IV/VI), indicating severe mitral regurgitation and a high likelihood of cardiogenic PE. By contrast, the dog in the case 2 showed a normal heart rate and softer left apical murmurs, suggesting subclinical MMVD so dyspnea was unlikely to be due to cardiogenic PE.

Cardiogenic PE develops secondary to the high diastolic LV pressure which transmits to the LA, the pulmonary veins, and the pulmonary capillaries (Kellihan et. al., 2015). In the case 1, the dog exhibited LA enlargement and pulmonary venous engorgement which were evident on the chest radiograph. In tandem with the above findings, the echocardiogram also showed a markedly dilated LA and a mildly enlarged LV chamber. The lower trans-mitral E wave velocity (<1.2 m/s) was most likely attributable to abrupt preload reduction by the repeated high-dose furosemide treatments (Courtois, et al., 1988), although the furosemide also reduced left atrial pressure (Suzuki et. al., 2011) in spite of the finding of wet lungs. Although the dog showed improvement after the diuretic therapy, the PE did not resolve completely as shown by the multiple B-lines visible on the lung ultrasound. In circumstances where thoracic radiography is not possible due to patient distress, lung ultrasonography diagnoses PE with a sensitivity of 90% and a specificity of 93% (Vezzosi et. al., 2017).

The occurrence of PE due to PH is an uncommon finding in veterinary literature. Kellihan et. al., (2015) speculated that the PE in the PH dogs arises from focal over-perfusion of capillary beds. In the poorly constricted lung region, the pulmonary capillaries have higher hydrostatic pressure but poor alveolar membrane integrity, resulting in protein- and erythrocyte-rich edema formation. In the case 2, the presence of pulmonary alveolar infiltrates provided a strong evidence of PE, whereas the echocardiography suggested a high-probable PH in the dog. Although the underlying cause for the PH remained questionable, the cardiology examination ruled out heartworm disease, left-sided heart failure, and cardiac shunts which are known to contribute to the development of PH in dogs. In addition, the PH was believed to be associated with chronic respiratory disease from the history of chronic coughing in the dog. We speculated that the chronic inflammation and hypoxia in the lungs resulted in non-regenerative anemia and left-shift leukocytosis. As the dog was a geriatric patient, neoplasm and hormonal diseases which are known to cause hypercoagulable state and pulmonary thromboembolism were not ruled out, although blood clot was not seen in the pulmonary artery on the echocardiogram. Further, the dog was also afebrile so infectious pneumonia was unlikely. Nevertheless, the dog clinically responded to the sildenafil and oxygen treatments, which represent the cornerstone therapy for pulmonary hypertension-associated pulmonary edema (Kellihan et al., 2015). Regretfully, the cause of death in the dog was unknown without a post-mortem examination.

CONCLUSION

Dyspnoea or respiratory distress in dogs can be cardiac or non-cardiac in origin. Although MMVD is the most common heart disease in small-breed dogs, preemptive treatment with a diuretic agent in stabilising all dyspnoeic dogs should be avoided unless there is a strong evidence of cardiogenic pulmonary oedema.

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Case Reports

DEGENERATIVE MITRAL VALVE DISEASE (STAGE C) IN A CHIHUAHUA

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SUMMARY

A 10-year-old intact male Chihuahua dog was presented with a history of exercise intolerance, coughing with a gag, panting and syncope. Heart auscultation revealed the dog was having left apical systolic murmur grade IV/VI with radiographic findings of cardiomegaly, pulmonary oedema and bronchial collapse. Echocardiography confirmed the dog had degenerative mitral valve disease (DMVD) with echocardiographic findings of thickened mitral valve leaflets and severe mitral regurgitation. The dog was treated with angiotensin converting enzyme inhibitor (ACEI), diuretics and bronchodilator. The dog responded well to the treatment with no occurrence of syncope since the last episode and reduced in frequency of cough. Pimobendan was added on to the treatment six months later as the dog started to have productive cough. Improvement was seen after 2 weeks where episodes of productive cough were greatly reduced with improved appetite. The prognosis is fair to poor in this case due to signs of congestive heart failure (Stage C) that required a life-long treatment therapy.

Keywords: coughing, syncope, echocardiography, mitral valves, Chihuahua

INTRODUCTION

Degenerative mitral valve disease (DMVD) is the most common, progressive, acquired, cardiac disease in geriatric dogs (Abbott, 2016). Development of DMVD was believed due to genetic inheritance of polygenic trait that causes defect in the quality of valve connective tissue (Abbott, 2016). Changes in the valve morphology had been characterised by glycosaminoglycans accumulation, thickened sub-endothelium and fibrosis of the valve apparatus (Atkins *et al*., 2009; Haggstrom, 2010).

Recent study showed that DMVD has been association with alteration from fibroblast phenotype to smooth muscle cell phenotype (Black *et al*., 2004). The disease can affect any breed but its prevalence is greater in small to medium breed dogs less than 20 kg such as Poodle, Papillon, Cavalier King Charles Spaniel, Dachshund and Chihuahua (Egenvall *et al*., 2006; Atkins *et al*., 2009). Male dogs are more commonly affected than female (Atkins *et al*., 2009). Various designations have been proposed for DMVD over the years such as myxomatous mitral valve disease (MMVD) and mitral valve endocardiosis (Atkins et al., 2009; Haggstrom, 2010), but all referred to the same disease and condition.

This case report of DMVD diagnosed in a Chihuahua responded well with post-therapy of pimobendan with no syncope observed during the long term treatment regime.

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CASE REPORT

A 10-year-old intact male Chihuahua was presented with a history of exercise intolerance, coughing with gag and panting for almost one month. The dog had a first episode of syncope one week before presentation and the second episode occurred in the morning prior to presentation.

Upon physical examination, the temperature and heart rate were within normal limit and respiratory rate was not taken but the dog was panting excessively. The dog was bright, alert and responsive with body condition score 3/5. Vaccination and deworming status were up-to-date. Auscultation of the heart revealed a left apical systolic murmur grade IV/VI with differential diagnosis of degenerative mitral valve disease (DMVD), mitral valve dysplasia and dilated cardiomyopathy (DCM). Harsh lung sound was noted and the dog was dyspneic with a differential diagnosis of cardiogenic pulmonary edema, bronchopneumonia and bronchitis.

Thoracic radiography and echocardiography were planned for further investigation. On the left lateral view of thoracic radiograph (Figure 1), the vertebral heart score (VHS) was 13 with increased sternal contact of heart, suggestive of marked cardiac enlargement. There was dorsal elevation of left mainstem bronchus with bronchial collapse, secondary to enlargement of the left atrium, based on the rounded caudal border of the cardiac silhouette. Thus, a radiological diagnosis of left sided heart enlargement was made. Radiological diagnosis of cardiogenic pulmonary oedema was made based on the increased in the lung parenchyma density with interstitial pattern at left cranial and caudal lung lobes, especially at the hilar region. Ventro-dorsal view of thoracic radiograph (Figure 2) revealed cardiomegaly with a bulge

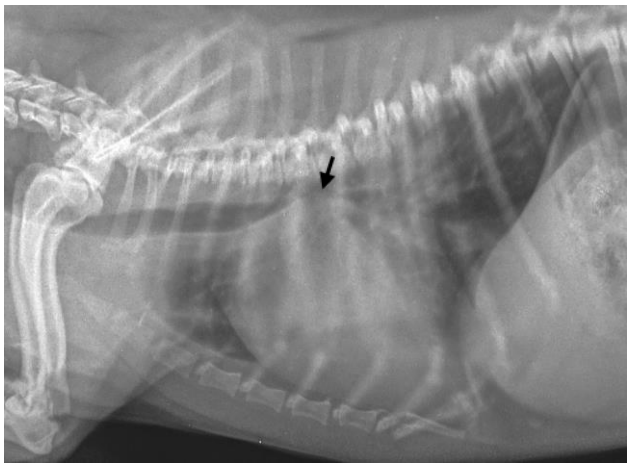

Figure 1. Left lateral view of thoracic radiograph showed marked enlargement of heart with VHS of 13. Left mainstem bronchus was elevated dorsally and there was bronchial collapse due to left atrial enlargement (black arrow). Lung parenchyma density increased at cranial and caudal lung lobes with interstitial pattern especially at the hilar region.

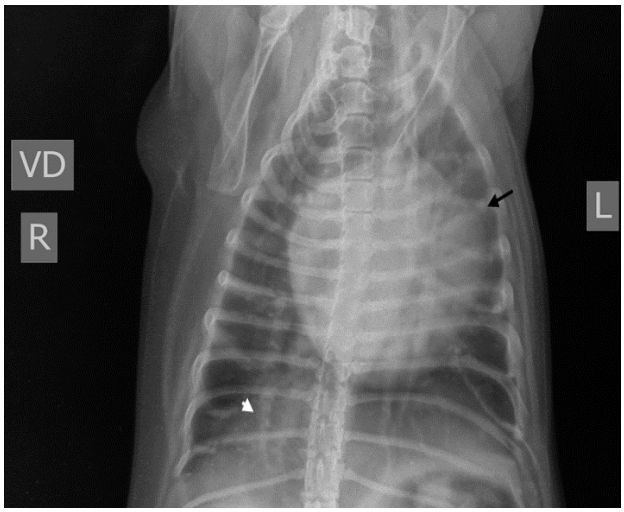

Figure 2. Ventro-dorsal view of thoracic radiograph showed cardiomegaly with a bulge at 2 to 3 o' clock position, consistent with left atrial enlargement (black arrow). There is a decrease in space between left cardiac outline and thoracic ribs. There was also bilateral increase in pulmonary parenchyma density with interstitial pattern and presence of right pulmonary vessel dilation (white arrow head).

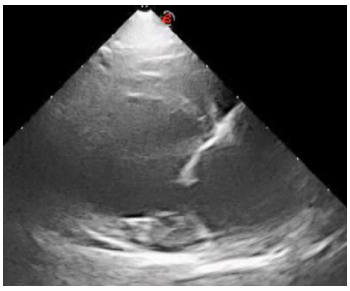

Figure 3. Right parasternal long axis four chamber view of echocardiography showed thickened mitral valve leaflets with club-shaped appearance at the tip.

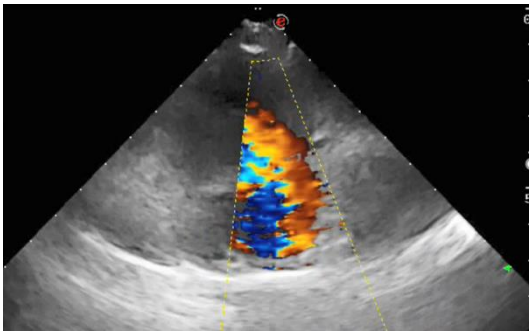

Figure 4. Left apical two chamber view with colour flow Doppler showed mosaic pattern, suggestive of regurgitation from left ventricle into left atrium.

at 2 to 3 o' clock position, consistent with left atrial enlargement that caused a decrease in space between left cardiac outline and thoracic ribs. There was also bilateral increased opacity in the pulmonary parenchyma with interstitial pattern which further support evidence of pulmonary oedema. The right pulmonary vein was dilated.

Echocardiography

On the right parasternal long axis four chamber view of echocardiography, the tips of the mitral valve leaflets appeared rounded with club-shaped appearance (Figure 3) which is suggestive of thickened mitral valve, that prevents proper leaflets alignment and closure. On left apical two chamber view, colour flow Doppler suggested presence of regurgitation from left ventricle into left atrium (Figure 4). Pulsed-wave Doppler mitral inflow was measured to evaluate diastolic function by determining the velocity of early passive filling at start of diastole (peak velocity of E-wave) and velocity of filling across mitral valve during active phase of diastole, that is atrial contraction (peak velocity of A-wave). Increased peak velocity of E-wave was suggestive of increased left atrial pressure consistent with a restrictive transmitral

Table 1. Parameter of mitral inflow and mitral regurgitation were collected from left apical two chamber view

Parameter	Result	Reference range
Mitral inflow		
E wave	1.40 m/s	0.30 – 0.90 m/s
A wave	0.62 m/s	0.30 – 0.60 m/s
E/A ratio	2.26	0.98 – 1.70
Mitral regurgitation	5.21 m/s	<1.70 m/s

E wave, peak velocity of E-wave; A wave, peak velocity of A-wave

Table 2. Parameter of LA/Ao ratio and M-mode measurement of left ventricular diameter and thickness were collected from right parasternal short axis view.

Parameter	Result	Reference range
LA/Ao ratio	2.06	<1.60
Cornell Normalised Reference Ranges	**Normalised Value**	**95% Confidence Interval**
Left Ventricle Diastole		
IVSd	0.41	0.29 – 0.59
LVIDd	2.06	1.27 – 1.85
LVPWd	0.44	0.29 – 0.60
Left Ventricle Systole		
IVSs	0.80	0.43 – 0.79
LVIDs	0.97	0.71 – 1.26
LVPWs	0.64	0.48 – 0.87

LVDd, Left Ventricular Diameter at Diastole; LVDs, Left Ventricular Diameter at Systole; IVSd, Interventricular Septal thickness at Diastole; IVSs, Interventricular Septal thickness at Systole; LVPWd, Left Ventricular Free Wall thickness at diastole; LVPWs, Left Ventricular Free Wall thickness at Systole; LV, left ventricle; Ao, Aorta

flow pattern whereby E/A ratio > 2.0. On the right parasternal short axis view at the aortic valve level, increased in left atrium to aorta (LA: Ao) ratio was suggestive of left atrial dilation. M-mode measurement of left ventricular diameter and thickness showed increased left ventricular diameter at diastole, suggestive of increased preload in left ventricle. Fractional shortening for myocardial contractility evaluation obtained was 46% (reference range: 25 – 50%) which indicated normal myocardial function. Based on the information obtained in addition to the history and presenting signs, the final diagnosis in this case was DMVD stage C.

The dog was treated with benazepril hydrochloride (Fortekor™ 5 mg, Elanco France), 0.5mg/kg once a day orally. Benazepril hydrochloride is a selective inhibitor of angiotensin converting enzyme (ACE) that acts as arteriolar and venous vasodilator and reduces sodium and water retention by kidney, thereby reduces afterload. Furosemide (LASIX®, Sanofi, Canada) a diuretic which was administered at 1mg/kg, twice a day orally was indicated for cardiogenic pulmonary oedema to decrease venous congestion and fluid accumulation. Furosemide is a loop diuretic that acts on the ascending loop of Henle to increase renal excretion of water along with sodium, potassium, chloride, magnesium, calcium, hydrogen, ammonium, and bicarbonate. Theophylline (Theolin® 200mg, Thailand) administered at 10mg/kg twice daily orally as a bronchodilator was indicated for bronchial collapse.

Heart condition of the dog was stabilized after treatment as the dog had coughed less frequently and there was no occurrence of syncope since the last episode. Second echocardiography was repeated after six months to evaluate the progression of heart condition. Left atrium to aorta (LA/Ao) ratio had increased from 2.06 to 2.64 which suggestive of further increase in left atrial size. Fractional shortening had decreased from 46% to 36% which suggested that there could have been a reduced myocardial contractility. Owner complained that the frequency of cough has gradually increased, productive cough was heard almost every day. Therefore, oral pimobendan (Cardisure® 5 mg, United Kingdom) at 0.5mg/kg prescribed twice daily was indicated to enhance myocardial contractility as well as to provide systemic and pulmonary vasodilatory effects. After 2 weeks of pimobendan, the dog was reported to cough only during excitement with improved appetite. Furosemide was then tapered down to 0.5mg/kg twice daily orally, as the dog had clear lungs sound and was maintained on both benazepril hydrochloride and pimobendan.

DISCUSSION

Heart failure can be defined as circulatory or myocardial failure involving functional or structural abnormalities which prevent proper functioning of the heart (Oyama, 2010). In heart failure patient, the compensatory mechanisms are activated in order to maintain normal cardiac performance but may consequently contribute to the establishment of congestive heart failure, particularly with the presence of oedema (Oyama, 2010). The abnormal fluid accumulation in the interstitium was due to an elevated capillary hydrostatic pressure secondary to an increased atrial pressure (Fuentes, 2010).

Myxomatous degeneration of the mitral valves occurs due to a hereditary defect in collagen synthesis and/ or remodeling of the valve apparatus. Non-inflammatory, progressive disarray of the valve structure arises thus affecting the mechanical integrity and proper apposition of the leaflets (Haggstrom, 2010; Madsen, 2011). The latter imposes further endothelial damage and causes an altered production of growth-inhibiting and growth-promoting substances of the subendothelial cells in the valve, also known as the valvular interstitial cells (Black *et al*., 2005; Mow & Pedersen, 1999). Subsequently, excessive glycosaminoglycan deposits and the transition of valvular interstitial cells to its myofibroblasts phenotype lead to further valves deformity (Black *et al*., 2005; Haggstrom, 2010). The resulting backflow of blood into the left atrium due to coaptation failure of the mitral valve leaflets, secondary to the aforementioned progressive valvular lesions is known as mitral regurgitation (Haggstrom, 2010). Mitral

regurgitation results in a loss in forward stroke volume thus activating the cardiac compensatory mechanisms, namely activation of neurohormonal pathways, increased cardiac contractility, eccentric hypertrophy, myocardial dilation and fluid retention (Haggstrom, 2004) in order to improve the mechanical environment of the heart. Consequently, these cause elevated cardiac preload thereby diminished cardiac output and increased venous pressures (congestion) especially pulmonary dysfunction. Left-sided congestive heart failure consequent to pulmonary venous hypertension develops with transudation of extracellular fluid into the alveoli and interstitial space, thus leading to pulmonary oedema. Common clinical signs of left-sided congestive heart failure include coughing, dyspnea, syncope, lethargy, exercise intolerance, cardiac cachexia and anorexia (Haggstrom, 2010).

The American College of Veterinary Internal Medicine (ACVIM) consensus guidelines is a classification system developed to allow better clinical approach towards diagnosis, management and treatment of degenerative mitral valve disease, whereby there are four stages. Stage A indicates dogs at higher than average risk for developing heart failure but without any apparent structural cardiac disorder (that is, no audible heart murmur) upon examination. Stage B indicates dogs with structural cardiac disorder without developing clinical signs of heart failure associated with their disease. It is further classified into Stage B1 and B2. Stage B1 identifies asymptomatic dogs with no diagnostic imaging evidence of heart remodeling while Stage B2 identifies asymptomatic dogs with evidence of heart remodeling via thoracic radiography and/ or echocardiography. Stage C indicates dogs with current or past clinical signs of heart failure due to DMVD that are not refractory to standard heart failure treatment. These patients may require in-house management (hospitalization) or managed as an outpatient. Stage D indicates dogs with clinical signs of heart failure refractory to standard treatment for Stage C. Thus, these patients require more than a total daily dosage of 8mg/kg furosemide or the equivalent dosage of torsemide, administered concurrently with standard doses of the other medications thought to control the clinical signs of heart failure. Anti-arrythmic medication may be needed to maintain sinus rhythm or regulate ventricular response to atrial fibrillation (Keene *et al.,* 2019). The dog in this case was classified under Stage C as it was identified as having past and current signs of congestive heart failure due to DMVD that required a long-term standard therapy for heart failure in order to remain clinically stable.

Follow up examinations are vital in monitoring of disease progression, enhancing treatment compliance and dosage adjustment (Atkins *et al*., 2009). Monthly blood tests were suggested for evaluation of renal function, i.e. serum urea and serum creatinine, and serum electrolytes as these parameters may be negatively influenced by medications used in the treatment of heart disease. Echocardiography was recommended every six months to evaluate for systolic function of the heart, severity of mitral regurgitation and changes in valvular lesions, representing the prognosis and disease progression. In addition, monitoring of hydration status was done via daily monitoring of water intake. Previous study showed the mean daily water intake for dogs was 71 mL/kg/day (Zanghi & Gardner, 2018). "Cyclical" appetite and cardiac cachexia are of concerns in patients with refractory congestive heart failure, thus adequate protein and calorie intakes are essential (Freeman & Rush, 2012). At least 5.14 g/100 kcal of high-quality protein and 60 kcal/kg of daily calorie intake should be fed to dogs with congestive heart failure to minimize loss of lean body mass. Dietary sodium should be restricted to less than 50 mg/100 kcal/day to prevent further congestion (Atkins et al., 2009; Freeman & Rush, 2012). Omega-3 fatty acids such as fish oil proved to have anti-arrhythmic and anti-inflammatory effects by reducing the production of inflammatory cytokines, thus preventing significant protein (muscle) catabolism and loss of lean body mass. It should be considered as a supplement to improve appetite and prevent cardiac cachexia (Freeman & Rush, 2012). Essential amino acids namely arginine supplement helps in the maintenance and improvement of endothelial function. Other dietary supplements such as co-enzyme Q10 and L-carnitine are essential for myocardial energy production with a recommended dosage of 100 mg/kg/day and 90 mg/day, respectively (Freeman & Rush, 2012). Available prescription cardiac diets are recommended to support general cardiovascular function.

Prognosis in this case was fair to poor as the dog was classified as a patient of DMVD Stage C which required long-term treatment in order to improve its quality of life. In general, the prognosis for dogs with degenerative mitral valve disease varies greatly according to several risk factors such as severity of mitral regurgitation, treatment response, myocardial function, presence of underlying systemic disease and development of complications from congestive heart failure (Haggstrom, 2010). The mean survival time for dogs with obvious clinical signs of congestive heart failure is 12 to 14 months and the rate of disease progression varies for every individual (MacGregor, 2014). 14 months since the first diagnosis was made, the dog in this case is currently maintained with the aforementioned dosages of pimobendan, benazepril hydrochloride and theophylline, besides the tapered dose of furosemide to 0.5mg/kg, once daily per orally. Bimonthly blood workup showed normal renal function. No recurrent sign of congestive heart failure has been reported to date.

CONCLUSION

Client education and owners` compliance are of great importance in achieving successful management of congestive heart failure (CHF). Despite incurable, degenerative mitral valve disease (DMVD) is a manageable disease whereby the treatment goals, diet and management of dogs with CHF are aimed at improving their quality of lives thus prolonging their lifespan.

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Case Reports

NEURALLY MEDIATED SYNCOPE IN A SHIH-TZU DOG WITH CONCURRENT STAGE B2 MYXOMATOUS MITRAL VALVE DISEASE

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SUMMARY

A 14-year-old female Shih Tzu dog was referred for determination of the cause of collapse. Initial diagnostic work-ups consisting of haematology, serum biochemistry, blood pressure measurement, chest radiography, and echocardiography did not reveal any abnormalities which could explain the collapse, except the stage B2 myxomatous mitral valve disease. The dog was treated with pimobendan but the problem did not resolve. Further investigations which were comprised of electrocardiography, vagal maneuver, and Holter's monitoring suggested an abnormal vagal tone in the dog. Since the treatment with theophylline, the dog did not experience any episode of syncope, thus confirming the diagnosis of neurally-mediated syncope.

Keywords: Neurally mediated syncope, vasovagal syncope, collapse, electrocardiography

INTRODUCTION

Syncope is the transient loss of consciousness and postural tone due to transient cerebral hypoperfusion (Davidow et al., 2001). The affected animals collapse suddenly, recover quickly, and are normal before and after the syncopal episode. In humans, the most common type of syncope is neurally mediated syncope (Mosqueda-Garcia et al., 2000), characterised by an inappropriate autonomic response to a stimulus, leading to bradycardia and hypotension (Willis et al., 2018).

Cough syncope, a form of situational syncope often occurs in dogs with brachycephalic conformation, airway disease, or myxomatous mitral valve disease (MMVD). In canine MMVD, the left atriomegaly and the concurrent bronchomalacia (Singh et al., 2012) precipitate the coughing, resulting in a transient increase in intra-thoracic and cranial pressures. Besides, coughing also stimulates vagal-mediated bradycardia and vasodilation. The combined outcomes include reduced cardiac output and cerebral perfusion, resulting in hypotension and syncope (Davidow et al., 2001).

CASE REPORT

A 14-year-old female Shih Tzu dog was presented for investigation of intermittent collapse. According to the owner, the dog collapsed 3 times over the past 3 weeks. The first episode occurred after it vomited whereas the second and third episodes occurred when the dog was in the cage. It remained conscious and recovered in less than 5 minutes. The dog also coughed occasionally. The videography recorded by the owner showed absence of twitching, urination, and defecation during and after the syncopal event in the dog. Review of the haematology and serum biochemistry results (Table 1) also did not show an electrolyte imbalance, hypoglycemia, or any other significant findings. Given the inconsistent clinical findings for seizures and the fact that the dog did not response to the treatment for seizures by the preceding veterinarian, differential diagnoses such as cough or cardiac syncope and neurally mediated syncope were made.

Table 1. Haematology and serum biochemistry results of this case.

Parameter (Unit)	Value	Reference
Red blood cell ($X10^{12}$/L)	6.12	5.50-8.50
Packed cell volume (L/L)	0.44	0.37-0.55
Hemoglobin (g/L)	142	120-180
White cell count ($X10^{9}$/L)	13.6	6.0-17.0
Platelet ($X10^{9}$/L)	284	200-500
Sodium (mmol/L)	151	138-158
Potassium (mmol/L)	4.6	3.8-5.8
Chloride (mmol/L)	103	100-115
Calcium (mmol/L)	2.63	2.10-2.80
Corrected calcium (mmol/L)	2.67	2.10-2.80
Phosphate (mmol/L)	1.82	0.87-2.10
Urea (mmol/L)	5.3	3.6-8.9
Creatinine (umol/L)	85	60-150
Albumin (g/L)	36	24-38
Globulin (g/L)	34	24-42
Alkaline phosphatase (U/L)	51.7	$\leq$68
Alanine transaminase (U/L)	35.3	10-72
Gamma-glutamyl transpeptidase (U/L)	49	10-80
Glucose (mmol/L)	5.53	4.4-6.6

On physical examination, the dog was bright and alert. Auscultation revealed heart rate (HR) of 132 bpm, sinus arrhythmia, and left apical systolic murmur grade IV/VI. Blood pressure (BP) measurement by oscillometry showed systolic BP of 127 mmHg, diastolic BP of 96

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mmHg, and mean BP of 106 mmHg. Chest radiography showed a normal heart silhouette (vertebral heart score: 9.5) (Figure 1) and mild broncho-vascular patterns (Figure 2). Echocardiography demonstrated thickening (Figure 3) and mild systolic prolapse of mitral valve leaflets, and moderate centric mitral regurgitation jets (regurgitant jet area to left atrial area: 40%). There was dilation of left atrium (LA) (LA to aorta: 1.81) (Figure 4) and left ventricle (LV) [LV diameter during diastole (LVDD): 29.4 mm; LVDD normalised for body weight: 1.7]. A diagnosis of stage B2 myxomatous mitral valve disease (MMVD) was made and the dog was treated with pimobendan (Cardisure 2.5mg, 0.19 mg/kg, PO, BID). The owner was told to monitor the dog for any relapse of collapse.

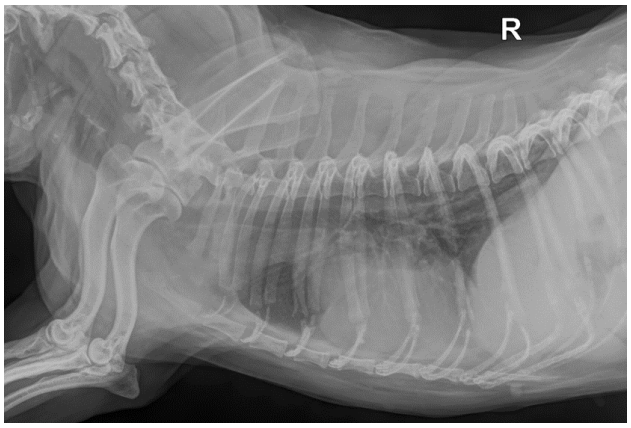

Figure 1. Right lateral thoracic radiograph shows a normal cardiac silhouette in the dog with barrel-shaped chest.

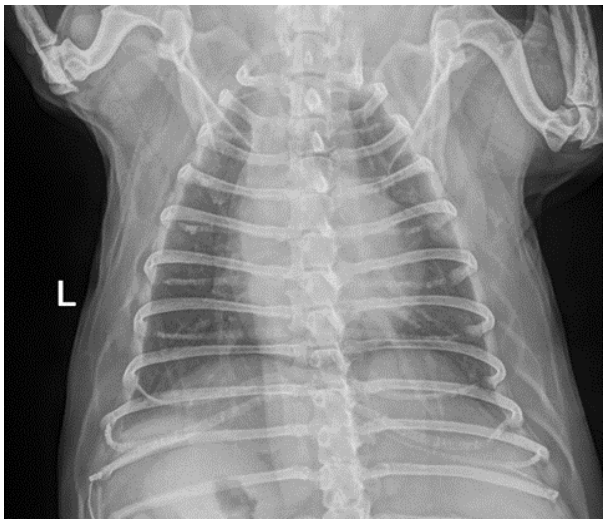

Figure 2 Ventrodorsal thoracic radiograph shows a normal cardiac silhouette and broncho-vascular lung patterns in the dog.

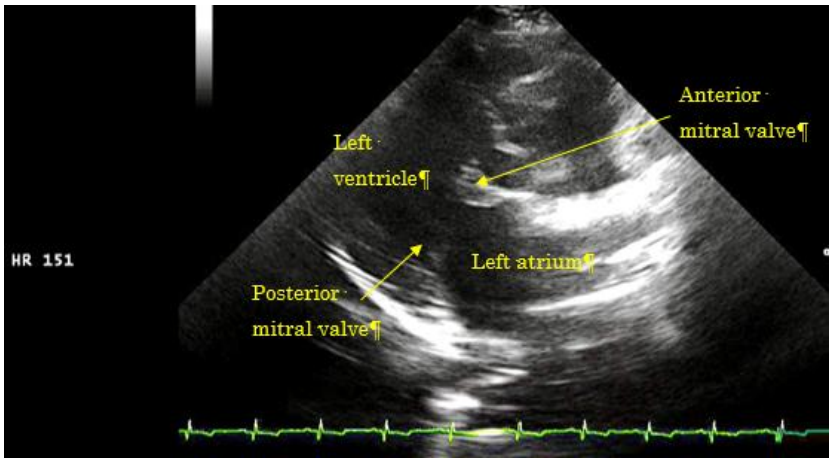

Figure 3 Right parasternal long axis view shows thickened anterior and posterior mitral valve leaflets.

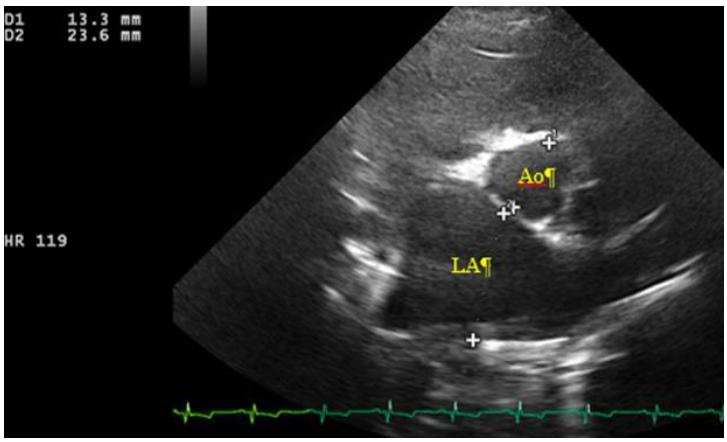

Figure 4 Right parasternal short axis view shows an enlarged left atrium (LA) compared with aorta (Ao) during end diastole. The LA:Ao ratio was 1.77, indicating left atrial enlargement.

Three days later the dog was presented for a revisit. On the day before presented, it collapsed once after it tried to turn its body position. An electrocardiography (ECG) was pursued. The resting ECG (Figure 5) showed heart rate of 132 bpm and sinus rhythm. Then, using a moist gauze, both eyes of the dog were closed and pressed for 10 seconds, and immediately the ECG recording was repeated. The ECG after the vagal maneuvers (Figure 6) demonstrated irregular RR intervals, heart rate of 123 bpm, and a sinus pause which lasted for 1.94 seconds. Next, a Holter's monitoring device was placed on the back of the dog for 24 hours. The owner was told to record the dog's activity at home and return the next day.

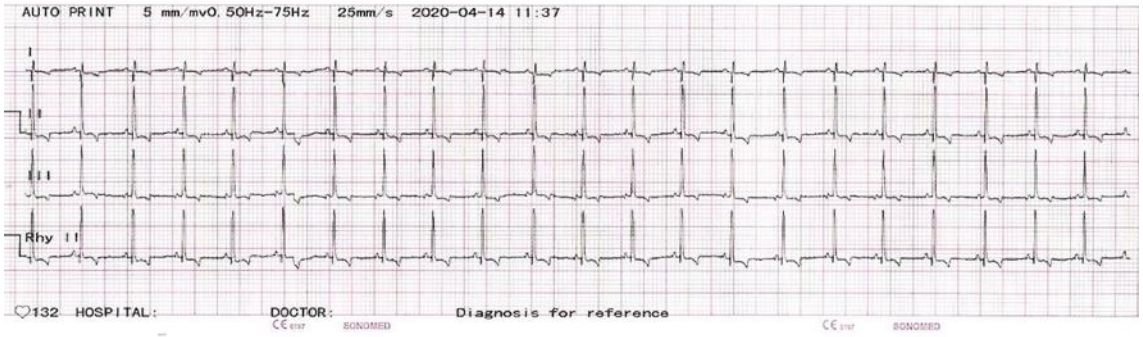

Figure 5. The resting electrocardiography.

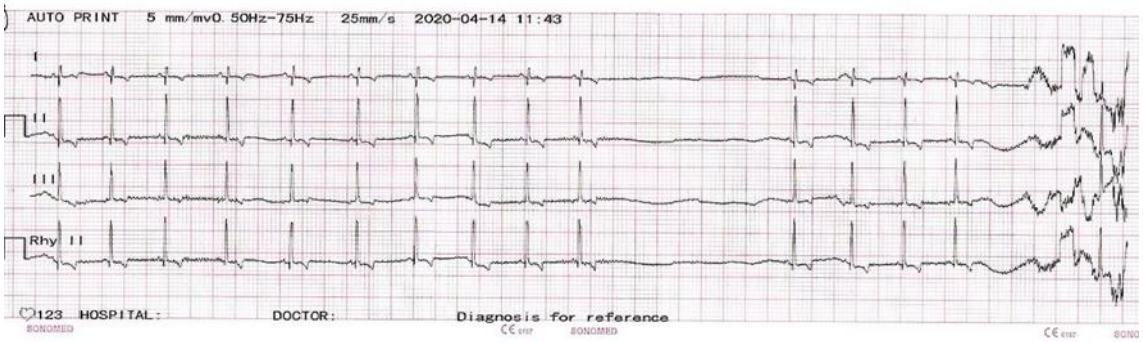

Figure 6. The post-vagal maneuvers electrocardiography.

A syncopal episode did not occur during the Holter's recording. The analysis showed average HR of 122 bpm, whereas the minimal and maximal HRs were 78 bpm and 187 bpm, respectively. Sinus pauses of more than 3 seconds were observed occasionally when the dog was sleeping (Figure 7), suggesting a high resting vagal tone. Interestingly, a longer pause of 4 seconds terminated by a junctional escape beat occurred when the dog was playing with other dogs in the morning (Figure 8). This showed that the dog had an abnormally high vagal stimulation during activity and excitement. In addition, neither brady- nor tachy-arrhythmia was observed during the 24-hour period. Therefore, a tentative diagnosis of neurally mediated syncope was made. Treatment with theophylline (TheoDur 100mg, 7.6 mg/kg, PO, BID) was started. Since then for about 5 months, the dog did not experience syncope and became more active.

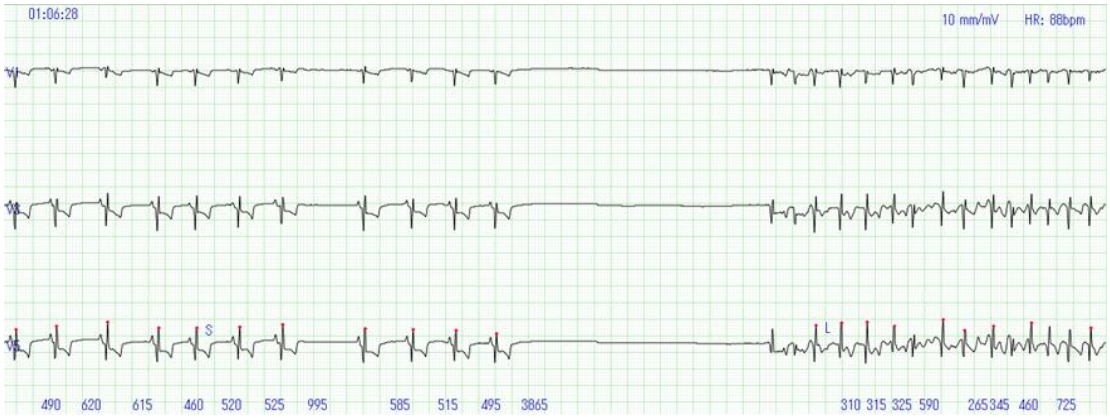

Figure 7. Holter's analysis when the dog was sleeping shows bradycardia, followed by a sinus pause of 3.3 seconds and a junctional escape beat.

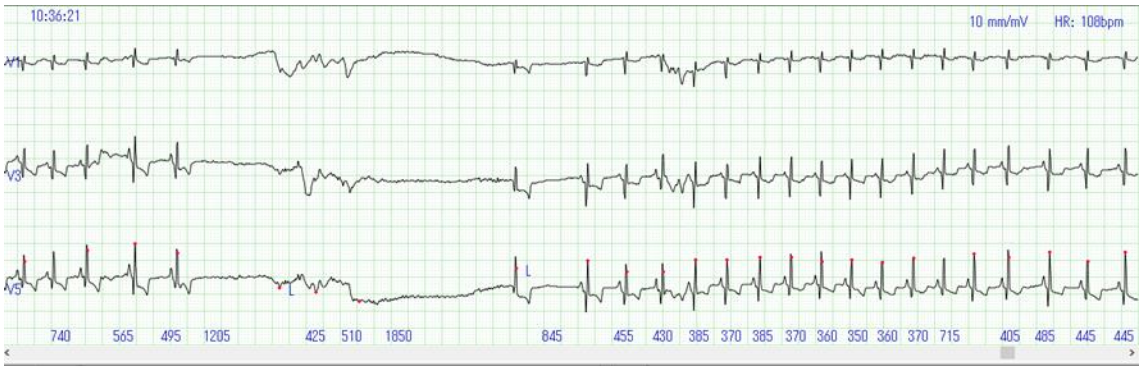

Figure 8. Holter's analysis when the dog was playing shows progressive bradycardia, followed by a sinus pause of 4 seconds, terminated by a junctional escape beat.

DISCUSSION

In the present case, the dog was presented with intermittent collapses due to neurally mediated syncope (NMC). Cardiac syncope was ruled out from the echocardiography as there was absence of stenosis (subaortic or pulmonic), intra-cardiac shunt, cardiomyopathy, and pulmonary hypertension. In severe myxomatous mitral valve disease, the enlarged left atrium may cause impingement on the main stem bronchi. This results in cardiac cough and subsequently syncope (cough syncope), although Singh et al. (2012) debated that the left atriomegaly alone without concurrent tracheobronchial disease may not be sufficient to cause cough. With this respect, the dog had neither severe left atriomegaly nor bronchial main-stem compression despite the diagnosis of stage B2 myxomatous mitral valve disease, so it had syncope unrelated to cardiac cough. Further, the dog experienced another syncopal episode even after the pimobendan treatment.

To elucidate the mechanism of syncope, Moya et al. (2009) proposed a classification system based on different electrocardiographic findings, which include asystole, bradycardia, tachycardia, and no or slight rhythm variation. Electrocardiographically, the NMC is characterized by a progressive sinus bradycardia, resulting in sinus arrest or atrioventricular block followed by ventricular arrest. In the present case, although the Holter's recording did not capture a syncopal event, it provided insightful information of unusual sinus arrest that was preceded by bradycardia during when the dog was presumably sympathetic-driven (playing and exercising). Further, the clinical history of the syncope after vomiting and a change in body positon also supported the diagnosis of NMC. It was speculated that the dog collapsed from failing to respond appropriately to trigger stimuli. The increased vagal tone resulted in profound hypotension from the cardio-inhibitory bradycardia and asystole (Moya et al., 2009).

In the present case, the dog was treated with theophylline, a methyl-xanthine derivative which inhibits phosphodiesterase and prostaglandin production, regulates calcium flux and intracellular calcium distribution, and antagonizes adenosine (Curry et al., 1985). By taking advantage of its inotropic properties, the heart rate and systemic blood pressure were increased, thus preventing profound hypotension and syncope in the dog.

CONCLUSION

In dogs, differentiating syncope from seizures can be challenging. A careful history taking and a thorough examination are required to investigate the cause of syncope.

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Case Reports

HEPATOCELLULAR CARCINOMA IN A MINIATURE SCHNAUZER

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SUMMARY

A 14-year-old spayed female Miniature Schnauzer diagnosed with liver cancer was presented for a second opinion. Upon physical examination, the temperature was normal with pale mucous membrane and a distended abdominen with enlarged liver was palpated. Abdominal ultrasonography revealed one single mass of the left liver lobe. Pre-anesthetic screening of the blood result revealed non-regenerative, normochromic, and normocytic anaemic with the elevation of liver enzyme. A complete left lateral liver lobectomy was performed and the excised liver was examined. A solid mass sized approximately 6 cm x 4 cm was found at the tip of the left lateral liver lobe. Histopathological examination of the mass revealed a well-differentiated hepatocellular carcinoma. There was no recurrence or metastasis detected for post-11-month surgery.

Keywords: miniature schnauzer, ultrasonography, lobectomy, hepatocellular carcinoma, metastasis

INTRODUCTION

The liver is the largest parenchymal organ in the body which is composed of the right medial lobe, right lateral lobe, caudate lobe, quadrate lobe, left medial lobe and left lateral lobe (Mauragis, 2016). The liver tumour can be of the primary tumour (originating from liver or hematopoietic system), or metastatic tumour (mostly arise from spleen, pancreas, or intestinal organ) (Alan *et al.*, 1995; Brooks, 2009; Ryan Hospital, n.d). Hepatocellular carcinoma (HCC) is the most common liver tumour amongst all other liver tumours such as mesenchymal tumour, neuroendocrine tumour, and bile duct tumour. For HCC, there are three morphological features of liver tumour which are massive, nodular, and diffuse (Bray, 2011). In this case, a massive liver tumour was detected, which was defined as a large tumour affecting a single lobe (Bray, 2011).

CASE REPORT

A 14-year-old 8 kg spayed female Miniature Schnauzer was diagnosed with liver mass based on ultrasonography and blood liver profile. The dog was presented for a second opinion for surgery. The owner noticed the dog was bloated with appetite reduced. Upon presentation, the dog appeared bright, alert and responsive with normal vital parameters. The abdomen was distended and felt doughy upon palpation. An apical systolic murmur of grade II to III out of VI was auscultated.

A complete blood haemogram revealed normochromic, normocytic, and non-regenerative anaemia. Serum biochemistry panels revealed elevated alkaline phosphatase (ALKP) with aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) were within the normal range (Table 1). Based on the echocardiographic investigation, the dog was diagnosed with degenerative mitral valve disease stage C. It was then revealed that the dog has been on heart medication for one year and hence the symptoms of the heart disease were managed well with a long-term medication using benazepril hydrochloride 5mg (Fortekor, Canada).

Abdominal ultrasonography (Esaote, Italy) showed massive heterogenous echogenicity liver mass, measured 5.84 cm x 4.14 cm on the left liver lobe with irregular margins (Figure 1). The gall bladder was distended with sludge and adherent to the wall. The spleen was small but with heterogeneous echogenicity. The structure of the left kidney shown an irregular cortico-medullary junction

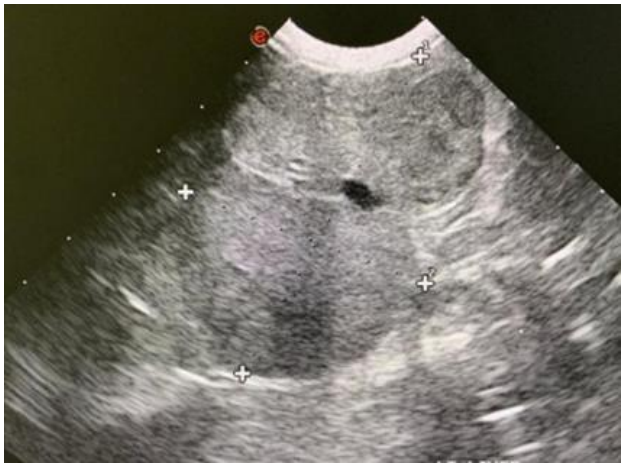

Figure 1. Sagittal plane view of mass at left liver lobe imaged obtained via ultrasound.

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Table 1. Haemogram and serum biochemistry results of this case before surgery and continuous monitoring post-surgery.

Blood parameter	Reference range	Pre-operative	Post-operative			
			Day 11	Day 57	Day 180	Day 257
Haemogram						
Red Blood Cell, RBC ($x10^6/mm^3$)	5.50 – 8.50	5.46	4.81	5.17	6.42	6.64
Haemoglobin, Hb (g/dl)	15 – 20	12.10	10.40	10.50	13.70	14.00
Packed Cell Volume, PCV (%)	44 – 57	33.60	24.20	33.10	39.50	40.90
Mean Corpuscular Volume, MCV (μm^3)	60 – 77	62	50.30	64.00	61.50	62.00
Mean Corpuscular Haemoglobin Concentration, MCHC (g/dl)	31 – 38	35.80	43	31.80	34.70	34.10
Serum Biochemistry						
Alkaline phosphatase, ALKP (U/L)	10 – 120	665	443	390	155	136
Alanine aminotransferase, ALT (U/L)	5 – 80	69	91	83	69	48
Aspartate aminotransferase, AST (U/L)	10 – 80	58	80	57	76	54
Gamma-glutamyl transferase, GGT (U/L)	1-10	3	13	8	3	0

whereas the right kidney had a slightly normal cortico-medullary junction.

The patient was pre-medicated with tramadol 3mg/kg (Tracidol, Malaysia), intravenously (IV) and induced with propofol 5mg/kg, IV (Troypofol, India). Then the patient was intubated and maintained with Isoflurane 3% (Isoflurin 1000mg/g, Spain). Midline exploratory laparotomy was made. The left liver lobe was isolated out from in abdominal cavity (Figure 2a).

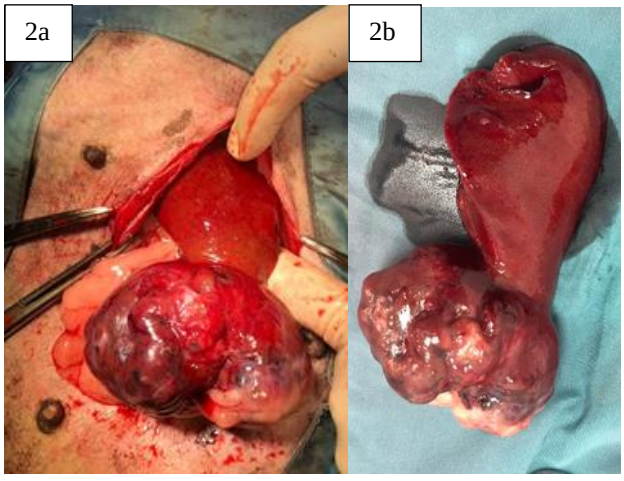

Figure 2a: (Left) Left liver lobe with a solitary mass in-situ upon examination during surgery.
Figure 2b: (Right) A complete excision of the liver lobe with the mass obtained for gross examination.

The left lateral hepatic artery, left lateral hepatic vein and left lateral portal vein were ligated with PDS 2/0 (monofilament, polydioxanone, reverse cutting, Vital Sutures, USA) and the whole liver was excised with scalpel blade size 20 (Figure 2b). Routine closure of the muscle and subcutaneous layer with PDS 2/0 (monofilament, polydioxanone, reverse cutting, Vital Sutures, USA). The skin incision was closed with Nylon 2/0 (monofilament, non-absorbable suture, reverse cutting, Unik Surgical Sutures MFG. CO., Taiwan). The mass measured 6 cm x 4 cm grossly and was located in the left lateral liver lobe. The mass was fixed into 10% formalin for two days and sent for histopathological examination. The histopathological report revealed that the mass was composed of neoplastic cells arranged in variably sized trabeculae supported by a fine sinusoidal network, and the hepatic parenchyma is a moderately well-circumscribed, unencapsulated, moderately cellular neoplasm. The neoplastic cells had round to oval nuclei with moderately stippled chromatin and variably distinct nucleoli. The mitotic rate is three per 10 high power fields (Figure 3a and 3b). The histopathological diagnosis for this case was well-differentiated hepatocellular carcinoma (HCC).

The patient's recovery from surgery was good. For post-operative medication, the patient was on intravenous fluid, enrofloxacin 10mg/kg orally (Baytril, Korea) and carprofen 2.2mg/kg orally (Canidryl, Ireland) and liver supplement (Liv. 52 Forte, 1 tablet twice daily, Australia). The patient was then discharged on the fourth day post-operative. On day 11 post-operative, liver enzymes that increased were ALT, and GGT but ALP had decreased. On day 57 post-operative, both ALP and ALT had decreased. AST and GGT were all within the normal limit (Table 1). Metastasis check via thoracic radiography and abdominal ultrasonography revealed no evidence of metastasis, abdominal lymph nodes were all quiescent.

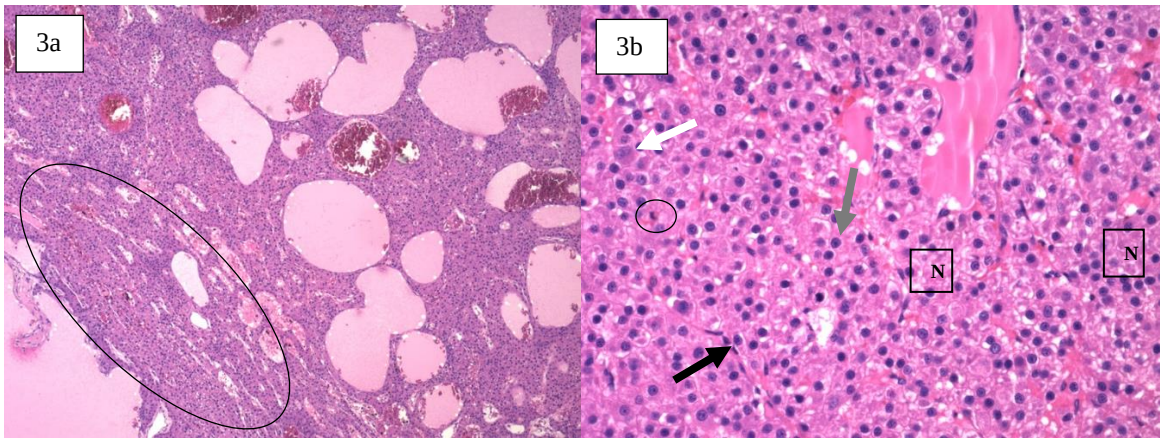

Figure 3a (left). The hepatic parenchyma is a moderately well-circumscribed, un-encapsulated cellular neoplasm. The neoplastic cells are arranged in variably sized trabeculae. The trabeculae are encircled (black). The neoplastic cells are supported by a fine sinusoidal network.
Figure 3b (right). Neoplastic cells are polygonal with often distinct cell borders and moderate amounts of eosinophilic cytoplasm that are sometimes vacuolated. The nuclei are round to oval with moderately stippled chromatin (grey arrow) with variably distinct nucleoli. Anisocytosis and anisokaryosis are moderate with occasional scattered karyomegaly, binucleates (white arrow). Presence of sinusoidal network with endothelial cells (black arrow) and mitotic figure (encircled in black) with the mitotic rate of three per 10 high power fields. There are multifocal areas of necrosis (N).
(Figure 3a: Hematoxylin and Eosin, 40x; Figure 3b: Hematoxylin and Eosin, 200x)

DISCUSSION

Primary liver tumours are rare and uncommonly reported in canine, only accounted for 0.6% to 1.5% amongst all canine tumours (Patnaik *et al.*, 1981). Conversely, metastatic tumours are more commonly seen and reported at two and a half times more compared to the primary liver tumour (Liptak *et al.*, 2004). Besides, due to the anatomy of the vascular and lymphatic, the liver is a common site for metastasis for extrahepatic neoplasms (Charles *et al.*, 2021). The most common primary liver tumour is HCC compared to other liver tumours such as bile duct tumours, neuroendocrine tumours (also known as carcinoids), and mesenchymal tumours (sarcomas) (Patnaik *et al.*, 1980; Sharon, 2009).

In this case, the gross morphological of the tumour was a massive liver tumour, which was defined as a massive solitary mass confined to a single liver lobe. This tumour is further defined based on morphology (2 types) namely either nodular or diffuse. Nodular is described as multifocal and would involve several lobes. Diffuse is defined as multifocal or coalescing nodules in all liver lobes, or diffuse effacement of the hepatic parenchyma (Patnaik, 1980; Brooks, 2009). The diagnosis of this case was based on the findings obtained from the ultrasound and liver biopsy. A fine-needle aspiration (FNA) and cytology are considered as less invasive approach than liver biopsy for obtaining a definitive diagnosis for HCC (Wypij *et al.*, 2006), but was rejected by the owner in this case due to the risk of excessive haemorrhage explained.

According to Patnaik *et al.* (1980), the average age of dogs diagnosed with HCC was 11 years old, and 80% were older than 10 years. In this case, the patient was 14-year-old. Interestingly, there was a case reported of HCC in a 25 month-old castrated male dog (Teshim *et al.*, 2013). There was no breed predisposition reported for hepatic neoplasms (Patnaik *et al.*, 1980; 1981). The common clinical signs reported include anorexia, lethargy, vomiting, hepatomegaly, ascites, jaundice, and/or weight loss. Seizure is rare and it might be caused by hepatoencephalopathy, paraneoplastic hypoglycaemia, or metastasis to the brain (Patnaik *et al.*, 1980). The patient showed signs of anorexia, lethargy, enlarged abdomen due to hepatomegaly, and slight weight loss.

As for the abnormal clinicopathological findings of the blood, anaemic was reported as the most common paraneoplastic syndrome in the majority of the tumour cases. This might be due to the consequence of chronic inflammation or infection causing an increase of inflammatory cytokines and chronic iron deficiency (Mellanby, 2011). In this case, the patient showed a mild, non-regenerative anaemia due to the chronic inflammation due to the liver mass. There are several liver enzymes categorized into inducible liver enzymes (ALP and GGT), and hepatocellular leakage enzyme (AST and ALT) (Lucia *et al.*, 2009). Elevation of liver enzymes were commonly reported such as elevated serum ALT, ALKP, AST, and/or GGT was as a result of hepatocellular damages or injures. Besides, the AST to ALT ratio (AST:ALT) may be useful for a further diagnostic workout to give a suggestive diagnosis. A ratio of AST: ALT of less than 1 was reported suggestive of HCC or bile duct carcinoma whereas if AST:ALT ratio was high (>1) could then be suggestive of neuroendocrine disease or mesenchymal tumours (Patnaik *et al.*, 1980). In this case, there was only an elevation of ALKP with AST:ALT ratio of 0.84 may be suggestive of HCC. Based on the post-operative serum ALKP level obtained, it was observed significantly reduced and the serum AST and ALT had remained normal. This indicated a good prognosis for the dog. During follow-up (day 257), ALKP is still slightly high compared to the normal range (Table

1). According to Lucia (2009), increased cholestatic liver enzyme activity is associated with several breeds such as Scottish terrier, miniature schnauzer, and Siberian husky. Serum alpha-fetoprotein (AFP) a tumour marker also has been used to diagnose HCC. A study showed that 4 dogs with HCC, 3 dogs with well-differentiated HCC had an elevation of serum AFP and was noted rapidly decreased after removal of the tumours from the dogs (Kitao *et al.*, 2006), but this test was not performed in this case.

In the present case, the massive HCC was involved in the left lateral liver lobe. The precedence of massive HCC involved in left lobes has been reported and stated as high as 68.3% compared to the right and central liver lobes (Liptak *et al.*, 2004). It was documented that dogs with massive left liver lobes have a good prognosis compared to right liver lobes. This might be due to the anatomical structure of the blood vessels (caudal vena cava) is intimately associated with the right-sided liver lobes (Liptak *et al*., 2004). Hence, this may explain the higher mortality rate intra-operative of the right-sided liver lobes compared to the left-sided liver lobes (Liptak *et al.*, 2004). A further diagnosis such as computed tomography is often recommended to determine the surgical approach. This advanced modality can delineate the relationship between the mass, blood vessels (caudal vena cava), and the liver lobe involved (Liptak *et al.*, 2004) and especially if the mass involved the right-sided liver lobes. It was recommended but not carried out in this case due to cost and was proceed with exploratory laparotomy.

For the treatment approach, surgical resection in solitary or massive HCC was often recommended. In this case, a complete left liver lobectomy was performed. According to Liptak *et al*. (2004), surgical resection of the left massive HCC resulted in a median survival time (MST) greater than 1460 days. A left liver lobectomy for massive HCC was reported with a better MST compared to the right-sided HCC. Distant metastasis rate was found to be as low as 4.8% and up to 36.6% of those cases with a massive lesion of HCC (Patnaik *et al*., 1981 and Liptak *et al*., 2004). In comparison between massive HCC of nodular and diffuse type, the prognosis of nodular HCC is better. Chemotherapy has been reported inefficient in the management of non-surgically resectable primary such as nodular, or diffuse HCC, and metastatic liver tumours (Bray, 2011). However, palliative intervention attempted may delay the progression of the disease (Bray, 2011).

For post-operative or follow-up approach, serum biochemistry, metastasis checks included 3-views thoracic radiography, and abdominal ultrasonography examination every 3 months for the first year (12 months) and then every 6 months thereafter (Liptak *et al*,. 2004). In this case, a metastasis check was performed on day 341, and revealed no distant metastasized on thoracic radiography, and abdominal ultrasonography. In this case, the prognosis is good as completed resection of the mass as well as there is no sign of metastasis till up-to-date.

CONCLUSION

HCC is the most frequent primary malignancy of the liver tumour. Massive HCC usually gives a better prognosis compared to a nodular, or diffuse HCC. Surgical resection of either complete lobectomy or partial lobectomy is recommended. In this case, the clinical outcome was good, as there was no local recurrence or distant metastasis after post-operative surgery resection.

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Short Communications

EFFECT OF CRYOPRESERVATION ON CELL VIABILITY AND T CELL FREQUENCY OF FRESH VERSUS FROZEN FELINE WHOLE BLOOD (WB) AND PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) SAMPLES

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SUMMARY

The cryopreservation of either whole blood (WB) or peripheral blood mononuclear cells (PBMC) is crucial to preserve cell viability in long term immunological studies. Flow cytometric analysis of lymphocyte subsets is of great importance and provides extra information in determining disease progression. In this study, cell viability and T cell percentages were assessed from fresh and frozen WB and PBMC samples. Blood samples were obtained from 14 cats and PBMCs were isolated by red blood cell (RBC) lysis and ficoll-paque density centrifugation technique. Fresh samples were immediately assessed for cell viability using trypan blue exclusion technique and CD4 and CD8 T cell percentage by flow cytometric analysis, while the frozen samples were stored in storage media in -80°C freezer for seven days and assessed for the same parameters upon thawing. The results showed that both fresh WB and PBMC had similar percentage of cell viability; however, the cell viability was significantly reduced upon thawing. Similarly, CD8 percentage of frozen WB and PBMC showed significant decrease compared to the fresh samples. Significant reduction of percentage of CD4 was observed between fresh and frozen PBMC, but not from whole blood. Overall, the results revealed that fresh PBMC sample is the preparation of choice for the enumeration of lymphocytes; while frozen PBMC would be the alternative if immediate analysis is not feasible. Optimization of cryopreservation and thawing protocols are warranted for future assessment of feline lymphocyte markers.

Keywords: PBMC, cryopreservation, cell viability, T cells frequency, flow cytometry

INTRODUCTION

Cryopreservation is a method of preserving the function and properties of biological materials at an extreme low temperature in the presence of cryoprotective agent. At low temperature of below -120°C, most cellular metabolism is halted, and the penetration of cryoprotective agent such as dimethyl sulfoxide (DMSO) into cells prevents damage via intracellular ice crystal formation during freezing and thawing process (Gao & Critser, 2000). This method is widely used to store peripheral blood mononuclear cells (PBMC) that consist of monocytes, lymphocytes, and natural killer (NK) cells for the purpose of *in vitro* immunoassays such as lymphocyte proliferation, immunophenotyping, cytotoxicity, and intracellular cytokine assays in response to microbial stimulation, drug, and vaccine treatment (Weinberg, 2016). Large amount of samples can be stored for extended period of time, allowing assays to be performed in a single batch without the need of repeating the experiment, thus, avoiding interassay variability while reducing resources, costs, and time. There were limited studies utilizing cryopreserved feline PBMC; however, from these studies, the function and phenotype of frozen feline PBMC were maintained upon thawing et al., 2012). For example, monocytes from frozen feline PBMC were able to differentiate into macrophage-derived dendritic cells and responded to inactivated feline viruses and differentiates into antigen presenting cells (APC). The generated APC was able to stimulate the proliferation of CD8 T cells which was also originated from frozen PBMC and induced the production of IFN-γ (Vermeulen et al., 2012).

The additional advantage of freezing feline PBMC is that it can reduce the number of blood draw from a single cat, which can be difficult and stressful especially for ill cats. Depending on the age, weight, and health condition of the cat, ethical criteria may only permit small volume of blood to be drawn at a time even under anesthesia, which makes some immunoassay impossible to be performed. Therefore, a simple alternative method is introduced which utilize whole blood (WB) instead of PBMC for cryopreservation to minimize cell loss during the isolation of PBMC. This approach is very convenient and economical because it does not require trained technician or specialized facilities to conduct PBMC isolation (Stevens et al., 2007). Furthermore, acquiring large amount of samples in the field for blood banking is feasible for studies that require huge data sets such as epidemiological studies (Cheng et al., 2007). Despite the reported success of WB cryopreservation, the protocols used in different laboratories vary considerably in parameters which includes the anticoagulant used upon blood collection, time between sampling and freezing, concentration of cryoprotective agents, time and temperature of shipment and storage, and the buffer used prior during processing (Langenskiold et al. 2018;

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Paredes et al., 2018; Schindler et al., 2004; Stevens et al., 2007; Verschoor 2018). These defined protocols were optimal for only limited number of functional and phenotype studies of the laboratories' interest, and they may not be reproducible in other laboratories performing different assays. Therefore, although freezing of WB is relatively easier and cheaper to perform, the freezing of PBMC has more advantage because it essentially shares similar protocols across different laboratories and more variety of immunoassay can be performed using thawed PBMC.

Here, this study aims to compare the viability and phenotypic frequency of lymphocytes markers CD4 and CD8 in short-term cryopreserved PBMC versus fresh PBMC, and cryopreserved WB versus fresh WB of feline species. The methods described here were relatively cheap and quick as it did not involve storage in liquid nitrogen, and the buffer used for red blood cell lysis was readily available to make. The success of cryopreservation protocol described here was based on the high viability, and the CD4 and CD8 frequency of cryopreserved feline WB and PBMC relative to the fresh samples, as human lymphocytes from frozen WB and PBMC is one of the subsets that had high viability and surface marker expression upon thawing (Aziz et al., 2013; Verschoor & Kohli, 2018; Verschoor et al., 2018; Weinberg et al., 2009). The practicability of this method should allow future immunological studies in feline species to be simplified and reproducible.

MATERIALS AND METHODS

Sample Collection

The approval to collect blood samples from animals was obtained from the Institutional Animal Use and Care Committee (IACUC) of Universiti Putra Malaysia prior to sampling (UPM/IACUC/AUP-U015/2019). Fourteen shelter cats from different animal shelters in Selangor area were recruited with the consent from shelters' management. At the site of sampling, approximately 3 mL blood was withdrawn from the jugular vein of cats before immediately transferred into EDTA Vacutainer (Becton Dickinson, USA) and mixed gently. After travelling for about 30 – 60 minutes from the sampling site to the laboratory, equal volume of blood was separated for: 1) lysis, viability assessment, and immunostaining of fresh WB; 2) cryopreservation of WB; and 3) PBMC isolation using ficoll-paque (GE Healthcare Bio-Sciences, Sweden) gradient centrifugation method. The isolated PBMC was further divided to equal amount for the purpose of viability assessment, and immunostaining of fresh PBMC, as well as cryopreservation of PBMC.

Sample processing and cryopreservation of feline whole blood (WB) samples

Upon arrival of blood specimen to the laboratory, the WB was immediately mixed with DMSO to a final percentage of 10% (v/v) in cryogenic tube, inverted to mix and placed directly in -80°C freezer. This preparation of sample was designated as frozen WB. For fresh WB sample preparation, red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM disodium EDTA) was added to the cat's blood in ratio of 10:1. The mixture was incubated at room temperature for 10 mins. The sample was centrifuged at 2000rpm for 5 mins and the supernatant was discarded carefully. After that, the cells were then washed twice with phosphate-buffered saline (PBS). The lysis protocol was repeated if necessary to remove all traces of RBCs. The cells from fresh WB were then immediately assessed for cell viability and flow cytometric analysis.

Isolation and cryopreservation of peripheral blood mononuclear cells (PBMC)

PBMC was isolated following the standard gradient centrifugation method. Briefly, fresh WB was mixed with equal volume of phosphate-buffered saline (PBS) before layered carefully over the same volume of ficoll-paque in a 15 mL conical tube. The sample was centrifuged in a swing-rotor centrifuge at a speed of 1500 rpm for 25 minutes, and the buffy layer was carefully aspirated using micropipette into a new clean 15 mL conical tube, and washed three times using PBS. Before the final wash, PBMC suspension was separated into two different conical tubes, one for the analysis of fresh PBMC and the other for freezing the PBMC. After the final wash, the fresh PBMC was resuspended in 1 mL staining buffer (PBS, 2mM EDTA, 0.5% goat serum), assessed for cell viability followed by immunostaining. For cryopreservation, the washed PBMC was resuspended in freezing medium consisting of 45% fetal bovine serum (FBS), 45% RPMI medium, and 10% DMSO and mixed gently before transferring into cryogenic tube. The tube was immediately stored in -80°C freezer.

Determination of Cell Viability using Trypan Blue exclusion method

Cell viability was counted in a haemocytometer using trypan blue dye (Sigma, Munich, Germany) following published protocols (Strober, 2015). The percentage of cell viability was calculated based on the total number of live cells divided by the total number of all cells.

Thawing of frozen feline whole blood (WB) and peripheral blood mononuclear cells (PBMC)

The thawing for frozen WB and frozen PBMC was conducted after exactly seven days of storage in -80°C by immediate thawing in 37°C water bath. For fresh WB and thawed frozen WB, red blood cells were lysed using RBC lysis buffer as described above for 10 mins at room temperature. The cells were washed twice with 10 mL PBS by centrifugation at 2000 rpm for 5 mins. This lysis step was repeated to remove traces of RBC in the sample. After the washing step, cells were resuspended in 1 mL of staining buffer and counted using trypan-blue exclusion method to assess the viability. After thawing of frozen PBMC, the cells were washed twice with 10 mL RPMI by

centrifugation at 2000 rpm for 5 mins before resuspension in staining buffer. The viability of recovered PBMC was also assessed using trypan-blue exclusion method. The cells from each sample were adjusted to 10^6 cells per 100 µL in staining buffer.

Immunostaining and Flow Cytometry Analysis

The flow cytometric immunostaining for fresh WB and fresh PBMC was performed on the same day of sample collection. For every 100 µL of cell suspension, 1 µL of CD4-FITC (Southern Biotech, USA) and 1 µL of CD8-PE (Invitrogen, USA) were added into the samples and incubated for 30 minutes at 4°C. The cells were washed with 1 mL staining buffer for every 100 µL twice by centrifugation at 1500 rpm for 5 minutes before fixing in staining buffer containing 1% paraformaldehyde and stored in 4°C chiller overnight before flow cytometry analysis. The population of positive cells was determined by gating population of single stained cells similar to unstained cells. Data were acquired using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA) with 10,000 events obtained for each sample and analyzed using NovoExpress Software (ACEA Biosciences, Inc, San Diego, CA).

Statistical Analysis

The data was analyzed and tabulated using GraphPad Prism 8.2.1 (GraphPad Software, San Diego, California). The data for each group (PBMC and WB) was first assessed for normality using Shapiro-Wilk test. Since the data was not normally distributed, Mann-Whitney Test was used to compare the mean of the cell viability, CD4 and CD8 T cells percentages between fresh and frozen samples of WB and PBMCs.

RESULTS

Cell Viability

From the fourteen cats recruited, the average percentage of viable PBMC and WB after fresh isolation was 96% for both cell types. However, after freezing of PBMC for 7 days, there was a significant reduction in the percentage of viable PBMC to 80%. Remarkably, the percentage of viable WB after frozen for 7 days was reduced to about 31% (Figure 1).

CD4 Frequency

To determine whether the cryopreservation of PBMC and WB can preserve the CD4 receptor on the T cells, freshly isolated and frozen PBMC and WB were stained using anti-feline CD4-specific antibody and the percent of positive cells were determined using flow cytometry. Using fresh samples, the percentage of CD4 T cells in PBMC and WB were 30% and 17%, respectively (Figure 2). A significant decrease of CD4 T cells percentage to 12% was observed in 7-day-old frozen PBMC. Interestingly, there was no significant change of CD4 T cells percentage between fresh and frozen WB.

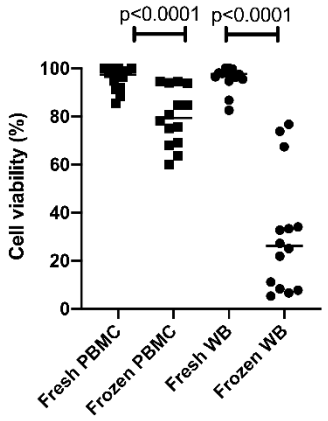

Figure 1. The percent viability of PBMC and WB after fresh isolation and frozen for 7 days (n=14). Data was represented as individual plot with mean and analyzed using Mann-Whitney test to compare the fresh and the frozen samples

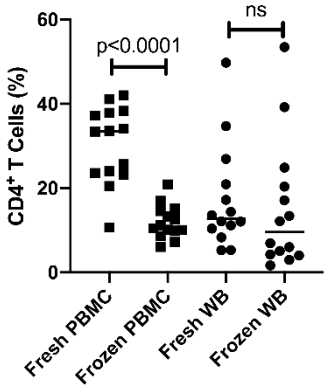

Figure 2. The percentage of CD4 T cells in PBMC and WB after fresh isolation and frozen for 7 days (n=14). Data was represented as individual plot with mean and analyzed using Mann-Whitney test to compare the fresh and the frozen samples.

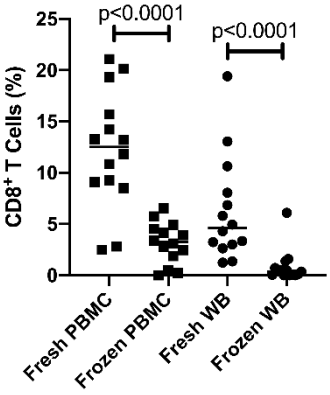

Figure 3. The percentage of CD8 T cells in PBMC and WB after fresh isolation and frozen for 7 days (n=14). Data was represented as individual plot with mean and analyzed using Mann-Whitney test to compare the fresh and the frozen samples.

CD8 Frequency

Similarly, the percentage of CD8 T cells in freshly isolated and frozen PBMC and WB were determined using flow cytometry. The results indicated that the percentage of CD8 T cells in fresh PBMC and WB was 12% and 6%, respectively (Figure 3). Both cell types showed significant reduction of CD8 T cell percentages upon freezing for 7 days, where the percentages were 3% and 1% for PBMC and WB, respectively.

DISCUSSION

The key for reliable and reproducible immunoassay data from cryopreserved PBMC or WB is the high percentage of cell viability post-thaw (Weinberg, 2016). Previous data has shown that a minimum of 70% of viable cells was required for frozen PBMC to obtain comparable result to fresh PBMC for lymphocytes proliferation and flow cytometry assays. The reason for this accepted threshold value was because a viability of less than 70% would introduce exogenous variables that can lead to the false interpretation of the host's immune response and phenotypic characteristics (Aziz et al., 2013; Weinberg, 2016; Weinberg et al., 2009). The method of cell viability and recovery assessment in these reports utilized trypan blue exclusion method, which is reliable and cheap method, although some laboratories used the automated cell counter such as Coulter Z1 automated particle counter (Beckman Coulter) which provides more efficient and consistent counting of live and dead cells (Aziz et al., 2013). The viable cell recovery was usually more than 60% of the number of fresh PBMC, but it has no influence of the function of lymphocytes compared to the fresh sample (Weinberg et al., 2009). The data presented here for feline PBMC cryopreservation was consistent with human PBMC cryopreservation, but there was a significant drop in the percentage of viable cells for frozen PBMC. Nevertheless, the percent viability of frozen PBMC for most cats tested were above 70% except for four cats (28.57%) which had lower than 70%. In contrast, there was a marked reduction of cell viability in frozen WB compared to fresh WB, where 11/14 cats (78.57%) had values of lower than 40%. These indicated that the cryopreservation protocol for both PBMC and WB need to be further optimized whereby the significant decrease of cell viability might arise from various sources during blood processing, freezing, storing, and thawing protocols.

A number of studies reported different usage of anticoagulant during blood collection such as acid-citrate-dextrose (ACD)-, EDTA-, and heparin-containing vacutainers. For cryopreservation of WB, treating the blood with ACD has the advantage of longer processing time for up to five days without compromising the viability of lymphocytes at room temperature compared to the other anticoagulants (Beck et al., 2001). However, several laboratories prefer to use heparinized or EDTA-containing vacutainer as it sustains the anticoagulant properties in the blood throughout processing unlike ACD which can introduce Ca^{2+} ions that may re-clot the blood when conducting the protocols (Alam et al., 2012; Stevens et al., 2007). Nevertheless, these anticoagulants were proven to show satisfactory results for flow cytometry assays for both frozen WB and frozen PBMC (Aziz et al., 2013; Langenskiold et al., 2018; Stevens et al., 2007; Verschoor et al., 2018). The optimal cooling rate for most cells types are typically around 1°C/min including for PBMC; however, different cell types may have different optimal cooling rates due to the differences in the permeability of plasma membrane and the components of cytoplasm that affect the movement of water in and out of the cells (Gao & Critser, 2000). For example, a cooling rate of 0.5°C/min was shown to preserve the surface marker and mitogen reactivity of T cells in PBMC when compared to cooling rate of 1°C/min (Milson & Keller, 1982). Interestingly, a cooling rate of 1°C/min for WB did not cause significant reduction in viability of lymphocytes and total PBMC in general, but monocytes population showed notable decrease in viability, while total leukocytes and neutrophils showed significant reduction in viability (Verschoor et al., 2018). This further supported that the optimal freezing rate of different cell types may be different, and the huge drop of viability observed in total leukocytes in frozen WB is accounted for neutrophils and monocytes, rather than lymphocytes (Verschoor et al., 2018). Therefore, the significant decrease in viability for frozen feline WB and PBMC observed here was most likely due to the osmotic shock from the rapid cooling and the high storage temperature in -80°C freezer. The optimal cooling rate reported for feline PBMC was 1°C/min until -30°C followed by incubation at -30°C for 15 minutes before further cooling at 1°C/min until -150°C and subsequent storing at -196°C liquid nitrogen (Vermeulen et al., 2012).

Despite the reduced viability and recovery of blood cell subsets in frozen WB, several reports found that the frequency of monocytes, T cells, and NK cells from cryopreserved WB were comparable to fresh WB, allowing the enumeration of these subsets using frozen WB (Alam et al., 2012; Langenskiold et al., 2018; Verschoor & Kohli, 2018). One study reported that the frequency of T cell subsets (CD3, CD4, CD8, CD27, CD28, and CCR7) in frozen WB were not significantly different from frozen PBMC after freezing for up to 120 days (Alam et al., 2012). In addition, the frequency of CD4 and CD8 T cell subsets from frozen WB were significantly correlated to fresh WB and the interassay variability of were not significantly different, however, this study did not compare the frequency between the two groups (Verschoor & Kohli, 2018). Interestingly, an earlier study showed that there was significant decrease in the frequency of monocytes, plasmacytoid DC, B cells, and basophils, in frozen WB compared to fresh WB, whereas a slight reduction of CD4 T cell count was observed but not in CD8 T cell count nor the resulting CD4:CD8 ratio (Verschoor et al., 2018). Collectively, this indicated that the frequency of T cells is more preserved compared to other PBMC subsets despite the drop in viability, which may explain the observation reported here where CD4 and CD8 T cells could still be detected from the frozen WB and frozen PBMC. The destruction of cell surface markers was also likely due to the rapid

cooling and high storage temperature. Besides that, the concentration of DMSO could also be the key to the prevention of intracellular ice formation during the cooling and thawing processes (Gao & Critser, 2000), whereby a slow dilution of DMSO-containing media with empty media after thawing can improve cell viability and recovery (Weinberg et al., 2009). Furthermore, the incorporation of feline serum may also improve the viability so that the lymphocyte marker can be salvaged (Azhar, 2016). Although it may be difficult to optimize the concentration of DMSO for WB due to inter-individual variability, a final concentration of 10% (v/v) DMSO and 30% - 90% fetal bovine serum in RPMI media is acceptable as freezing media for feline PBMC and WB (Vermeulen et al., 2012).

A few suggestions are proposed to better suit our purpose of cryopreserving feline PBMC for immunological assessment. The first is to utilize the TruCount technology, BD Trucount™ (BD Bioscience, USA) for the assessment of granulocytes, monocytes, and lymphocytes frequency in frozen WB, as one report showed that the frequency of the cell subsets were 91% to 94% of the fresh WB sample (Langenskiold et al., 2018). The advantage for this technology is that it is highly reproducible and only a small amount of WB (50 µL) is needed for the analysis for up to nine biomarkers, which is useful in feline immune studies when there is limited blood sample available. However, the disadvantage of this technique is that the viability could not be determined and the cost for each Trucount tubes containing the beads may be expensive. The second approach is to stain the WB with desired antibody prior to cryopreservation. This method was proven to be successful using a small volume of WB (100 µL) and the cells can be stored at -20°C for up to 30 days (Paredes et al., 2018), which is also an excellent option for feline immune assessment. The additional advantages of this method is that the cells can be stained using live cell staining for the measurement of cell viability, and the intracellular cytokines can also be stained upon thawing of cells (Paredes et al., 2018). The main disadvantage of this method is that the time of storage may not be too long compared to storage at temperature of below -80°C as the cells could possibly be deteriorating over extended period of time. The third suggestion is to apply the method of rapid thawing of the cells, even under a high cooling rate of the cells during freezing process. This approach is proposed because a recent study suggested that a rapid warming rate of between 45°C/min and 113°C/min significantly improve the viability of human peripheral blood T cells compared to a slow warming rate of 1.6°C/min and 6.2°C/min following a rapid cooling (Baboo et al., 2019). Although this method is convenient compared to other techniques, the correct range of warming rate may require optimization so that consistent result is obtain in different samples.

To conclude, this study provided new insights on practicability of using cryopreserved feline WB as a source of immune cells for analysis using flow cytometry. The methods described here requires significant improvement in the future so that the percent viability is enhanced, leading to reliable flow cytometric data and analysis.

CONCLUSION

This study shows that the cryopreservation of cell at -80°C had a significant effect on the cell viability and T cells percentage, thus the frozen samples would not be suggested as substitution of fresh samples for immunological study. Overall, the results revealed that fresh PBMC sample is the best sample for the enumeration of lymphocytes; while frozen PBMC would be the preparation of choice if long term storage is needed. In addition, further optimization of storage media and thawing protocols are needed for future assessment of feline lymphocyte markers.

CONFLICT OF INTEREST

None of the authors of this paper has financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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A	Ampere
A	alpha
ú	Angstrom
AAS	atomic absorption spectroscopy
AES	atomic emission spectroscopy
AFS	atomic fluorescence spectroscopy
a.m	ante meridiem
anhyd	anhydrous
AR	analytical reagent
atm	atmosphere
ATP	adenosine 5'triphosphate
ATP	adenosine triphosphate
at. wt.	atomic weight
AU	absorption units
av	average
β	beta
b.i.d/bid	twice a day
biol	biological(ly)
BOD	biological oxygen demand
bp	boiling point
BSA	bovine serum albumin
c	centi (10^{-2})
C	Celcius
C	coulomb
ca.	approximately
CAD	computer-assisted design
cal	calorie
calcd	calculated
cc	cubic centimetre (cm^3)
cfu	colony forming units (bacteria)
cm	centimeter
CoA	coenzyme A
coeff	coefficient
const	constant
cor	corrected
cpm	count per minute
cps	count per second (s^{-1})
cps	cycles per second (Hz)
Cq	threshold cycle
CPU	central processing unit
CPr	crude protein
cub	cubic
cwt	hundredweight
d	day
d	deci (10^{-1})
d	diameter
d	density
d	distance
da	deka (10)
dc	direct current
deg	degree
df	degrees of freedom
dil	dilute
distd	distilled
DV	dorsal ventral
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
e	electron
ECG	electrocardiogram
ed.	edition
Ed.	editor
Eds	editors
ED50	dose that is effective in 50% of test subjects
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
emf	electromagnetic force
emu	electromagnetic unit
equiv	equivalent
et al.	and others
etc.	and so forth
exp	exponential
expt(1)	experiment(al)
f	fento (10^{-15})
f	focal length
F	Faraday's constant
fp	freezing point
FSH	Follicle Stimulating Hormone
ft	foot
FTU	phytase activity
fw	formula weight
g	gram
gal	gallon
GC	gas chromatography
h	hour
H	henry
ha	hectare
Hb	haemoglobin
hp	horsepower
HPLC	high-pressure liquid chromatography
Hz	hertz
I	electric current ionic strength
ibid	in the same place
ic	intracerebrally
i.d.	inside diameter
i.e.	that is
im	intramuscularly
in	inch
ip	intraperitoneally
IR	infrared
IU	international unit
iv	intravenously
J	joule
k	kilo (10^3)
k	rate constant
K	equilibrium constant
km	kilometer
L	litre
lat	latitude
lb	pound
LD	lethal dose
LD50	dose that is lethal in 50% of test subjects
lit.	literature
log	logarithm to the base 10
long.	longitude
lx	lux
m	meter
M	mega
ME	metabolic energy
mg	milligram
mi	mile
min	minimum minute
ml	milliliter
mm	millimeter
mo	month
mol	mole
mol wt	molecular weight
mp	melting point
n	nano number
ng	nanogram
nm	nanometer
no	number
o.d.	outside diameter
OD	optical density
Oz	ounce
p	page
p	proton
p	pico (10^{-12})
Pa	pascal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
Pd	potential difference
pg	picogram
PFU	plaque-forming unit
pH	hydrogen ion concentration
p.i.	post inoculation
p.m.	post meridiem
po	per os
pp	pages
ppb	parts per billion
ppm	parts per million
ppt	precipitate
psi	pounds per square inch
psia	pounds per square inch absolute
psig	pounds per square inch gauge
pt	pint
qt	quart
R	resistance
rac	racemic
rd	rad (unit of radiation)
ref	reference
rel	relative
rf	radio frequency
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
sec	second
S	siemens
sc	subcutaneously
SD	standard deviation
SE	standard error
SEM	standard error of mean
SI	International System of Units
s.i.d/sid	once in a day
sp	specific
spp	species
sp gr	specific gravity
sp ht	specific heat
sp vol	specific volume
sq	square
std	STANDARD
t	temperature
t.i.d/tid	thrice in a day
temp	temperature
TSH	thyroid-stimulating hormone
u	micro
ul	microliter
um	micrometer
uhf	ulfra high frequency
UV	ultraviolet
V	volt
VD	ventral dorsal
viz.	namely
vol	volume
vp	vapour pressure
vs.	versus
v/v	volume to volume ratio
wt	weight